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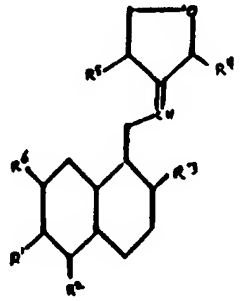
(54) Title: USE OF ANDROGRAPHOLIDE COMPOUNDS TO TREAT OR PREVENT PATHOGENICITY OF DISEASES

(57) Abstract

Disclosed herein is the discovery of a group of compounds that can cause expression and phosphorylation of $p34^{cdc2}$ kinase, cyclin B and Mos associated proteins to decrease within a cell or group of cells. Extracts of dried plants were assayed for the ability to decrease expression of $p34^{cdc2}$ kinase. An extract of *Andrographis paniculata* was discovered to have this effect. Later analysis showed that it is the andrographolide compounds in the extract that causes the reduction of expression of $p34^{cdc2}$ kinase. Andrographolide is known to have the following naturally occuring analogs: 14-epiandrographolide; isoandrographolide; 14-deoxy-12-methoxyandrographolide; 12-epi-14-12-methoxyandrographolide; 14-deoxy-12-hydroxyandrographolide; and 14-deoxy-11-hydroxyandrographolide. The compounds has structure (I) wherein: R1, R2 and R5 are one of the following: a hydroxyl group, a methyl group, a methoxy group, a methylene group, or an ether or ester linked sugar group; R3 is a methyl group or a methylene group; R4 is a hydroxyl group or a carbonyl group; R6 is hydrogen or a hydroxyl group.



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USE OF ANDROGRAPHOLIDE COMPOUNDS TO TREAT OR PREVENT PATHOGENICITY OF DISEASES

REFERENCE TO RELATED APPLICATIONS

This is a continuation-in-part patent application of a co-pending application serial number 08/349,989, filed December 6, 1994.

FIELD OF THE INVENTION

This invention relates to the use of andrographolide compounds to treat or prevent the pathogenicity of various diseases and more specifically this invention relates to the use of andrographolide compounds to regulate expression of the cell division control enzyme $p34^{cdc2}$ kinase, cyclin B and c-Mos.

BACKGROUND OF THE INVENTION

Over the past few years cell biologists have made remarkable progress in identifying the molecules that drive the cell cycle: the carefully choreographed series of events that culminates in cell division. In doing so they have not only provided a better understanding of one of the most fundamental of the cell's activities, they have also opened a new direction for research aimed at pinpointing the pathogenicity of cancer AIDS, Alzheimer's, angiogenesis and a variety of viral diseases associated with cancer. The reason for this intriguing convergence is that accumulating evidence indicates that derangements in the cell cycle machinery may contribute to pathology of a number of apparently unrelated diseases.

A family of cell division control enzymes termed cyclin-dependent kinase's (CDKs), along with the cyclin proteins, serves to control and coordinate the molecular events of cell division in all eukaryotic cells (Norbury and Nurse, 1992; Draetta, 1990; Draetta et al.1988; Bartlett and Nurse, 1990). Although twelve CDKs have been

described, $p34^{cdc2}$ kinase remains the most actively studied because of its central role in the control of cell division in both yeast and animal cells (Draetta, 1990; van den Heuvel and Harlow, 1993; Pines and Hunter, 1990; Norbury and Nurse, 1990).

In normal resting cells $p34^{cdc2}$ is not expressed or expressed at very low levels, but concentrations of $p34^{cdc2}$ increase as the cell enters and passes through G1 and the G1/S transition. $p34^{cdc2}$ concentrations reach maximal levels in the S, G2 and M phases (Loyer et al.1994). As used herein, the word "expression" refers to the level of active protein of any particular protein; expression of a protein may be affected by a variety of factors including changes in transcription, translation and protein catalysis.

In association with cyclin B, $p34^{cdc2}$ is the serine/threonine kinase subunit of M-phase-promoting factor (MPF); active MPF triggers the G2/M transition in species ranging from yeast to humans (Brizuela et al.1989; Draetta, 1990). Several studies also suggest that $P34^{cdc2}$ functions in the control of the G1/S transition and as well as the initiation of mitosis (Furukawa et al.1990; Krek and Nigg, 1991).

The role of CDK proteins is completely dependent upon their kinase activity in the cell cycle. $p34^{cdc2}$ kinase activity during the cell cycle is regulated primarily through post-translational modifications including cycles of phosphorylation and dephosphorylation (Ducommun et al.1991; Norbury et al.1991) and interactions with cyclins (Booher and Beach, 1987; Ducommun et al.1991; Williams et al.1992). Intracellular compartment translocation has also been demonstrated to regulate the substrate availability of the $p34^{cdc2}$ protein (Williams et al.1992; Pines and Hunter, 1991).

The functioning of $p34^{cdc2}$ involves the coordination of all events relating to cell division. In this role $p34^{cdc2}$ is the central information processing protein. As the cell moves through the cell cycle, information concerning the activities of the cell are sent to $p34^{cdc2}$ and as long as these signals indicate proper functioning of the cell, movement through the cell cycle continues. However, should information sent to $p34^{cdc2}$ indicate a problem with the cell (e.g. DNA damage, microtubule disruption) progression through the cell cycle would be halted. The block imposed on the cell cycle is at the G1/S interphase.

An example of the coordination of cellular events with progression through the cell cycle is given in Figures 1a and 1b. Figure 1a illustrates the increases of $p34^{cdc2}$ that are seen when the cell goes from a resting state into the cell cycle. Further increases in $p34^{cdc2}$ are seen when the cell transforms into a tumor cell. Figure 1b shows the role of protein expression in the feedback mechanisms involved in control of the movement of the cell through the cell cycle. The cellular expression of $p34^{cdc2}$ is governed by exposure to cytokines and hormones; the expression of $p34^{cdc2}$ is one signal to the cell to initiate the events of cell division. If the events of cell division are operating normally, $p34^{cdc2}$ levels will decrease (through specific proteolytic enzymes) and the cell will re-enter the resting state.

However, if the events of cell division are not functioning normally and $p34^{cdc2}$ concentrations in the cell remain elevated, cellular processes will be activated that block in cell at the G1/S interphase of the cell cycle. This block is mediated through the p53 protein (see Figure 1b) and involves the formation of a complex with p21 and $p34^{cdc2}$ that inhibits the kinase activity of $p34^{cdc2}$. The inhibition of the kinase activity of $p34^{cdc2}$ essentially blocks the flow of information from this coordinator of cell division and the cell remains in a G1/S stasis until concentrations of $p34^{cdc2}$ can be reduced and the cell enters a resting state.

A number of pathologies have been identified with the over expression of $p34^{cdc2}$ protein. Increasing evidence supports the relationship of aberrant $p34^{cdc2}$ expression and cancer (Yasui et al.1993; Pardee, 1989). Over expression of $p34^{cdc2}$ was noted in 90% of breast tumor cell lines examined (Keyomarsi and Pardee, 1993), in all 40 human cancer cells studied (Bartek et al.1993) and in all clinical gastric and colon carcinomas examined (Yasui et al.1993). Proliferation of vascular endothelial cells is mediated through $p34^{cdc2}$ expression (Zhou et al.1994); such stimulation is associated with angiogenesis and functions in the pathology associated with occlusion of arteries following trauma such as angioplasty (Morishita et al.1994).

$p34^{cdc2}$ is also implicated in HIV-1 envelope-mediated cell death. It has been demonstrated that during HIV-1 mediated cytopathogenicity CD4+ T-cells were killed by a mechanism involving functional $p34^{cdc2}$. Inhibition of the tyrosine phosphorylation of $p34^{cdc2}$ (a step performed in early G1) resulted in an inhibition of the killing of CD4+ T-

cells (Cohen et al.1993). Such a mechanism may also be involved in the cytopathogenicity of other viral diseases such as hepatitis or herpes.

Finally, $p34^{cdc2}$ may play a significant role in the pathology associated with Alzheimer's disease. Deposition and accumulation of amyloid A beta peptide in the cell is believed to be a primary biochemical defect in the ethology of Alzheimer's disease. Amyloid A beta peptide is derived from a larger precursor protein termed amyloid precursor protein (APP). Processing of APP is initiated through phosphorylation and is cell cycle dependent; this phosphorylation has been shown to be performed by $p34^{cdc2}$ both in vitro and in vivo (Suzuki et al.1994; Baumann et al.1993). These finding suggest that alteration in the kinase activity or expression of $p34^{cdc2}$ may attenuate the pathogenesis of Alzheimer's disease.

That finding that $p34^{cdc2}$ expression and activity relate to a wide variety of disease manifestations is rapidly becoming obvious as more research on the functions of this kinase is published. However for therapeutic purposes, the more direct question involves whether the manipulation of $p34^{cdc2}$ concentrations or activity in the affected cells alters cytopathology. During the last two years a body of research has been published indicating that it is possible to control cellular proliferation associated with cancer by inhibiting or downregulating $p34^{cdc2}$ (Lee et al.1994; Worland et al.1992; Losiewicz et al.1994; Worland et al.1993; Albers et al.1993; Jackman et al.1993; Jayaraman and Marks, 1993; Juan and Wu, 1993; Kitagawa et al.1993; Meijer et al.1993; Morice et al.1993b; Morice et al.1993a; Musgrove et al.1993). In addition to effects on a wide variety of tumor cells, the downregulation of $p34^{cdc2}$ has proven to be a successful mechanism to avert smooth muscle cell proliferation (Abe et al.1994b; Abe et al.1994a; Morishita et al.1994; Morishita et al.1994; Morishita et al.1993; Watson et al.1993).

That $p34^{cdc2}$ (i) exists in biological systems as diverse as yeast and humans, (ii) serves a fundamental role in control and coordination of the cell cycle, and (iii) is involved in a wide variety of pathologic consequences of abnormal expression, reflects the enormous importance of $p34^{cdc2}$ in cell physiology. Therefore the capacity of a chemical to affect $p34^{cdc2}$ concentrations in a multiplicity of cell types (e.g. epithelial, mesothelial) while avoiding direct cytotoxicity has profound therapeutic implications.

The c-mos proto-oncogene product, Mos, is a serine/threonine protein kinanse that controls the meiotic cell cycle in vetebrate oocytes. Constitutive Mos, c-Mos, and its viral counter-part (v-Mos) may play a role in oncogenesis. Studies have shown that mos acts through the mitogen-activated protein kinase (MAPK) cascade to induce cell transformation and that blocking the v-mos activation of that cascade prevents transformation. (Topol *et al.*, 1995)

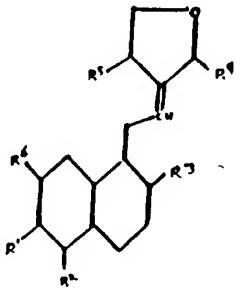
Other studies have shown that $p34^{cdc2}$ is complexed with the c-mos protein in rat skelatel muscle. (Leibovitch *et al.*, 1993) Faller *et al.* showed that v-mos suppresses platlet-derived growth factor (PDGF) type-beta receptor autophosphorylation and inhibits PDGF-BB-mediated signal transduction. (Faller *et al.*, 1994) These results suggest that Mos, or a similar serine/threonine kinase, has a role as a control mechanism in one of the earliest steps of the PDGF signal transduction paythway and provide a model for the functional interaction of Mos with growth factor receptors.

The plant *Andrographis paniculata* is widely used in China as a treatment of bacterial infections. The herb and various organic extracts of the herb have demonstrated antibacterial (Thamlikitkul et al.1991), and filaricidal (Dutta and Sukul, 1982) properties. Additionally, the herb has been shown to be antithrombotic (Zhao and Fang, 1990), and inhibit stenosis and restenosis after angioplasty in the rat (Wang and Zhao, 1993). Because of the widespread use of this herb, a number of chemicals have been identified as secondary metabolites. Research reports of diterpenoids with biological activity have been published. These diterpenoids have demonstrated hepatoprotective (Kapil et al.1993), immunostimulant (Puri et al.1993), choleretic (Shukla et al.1992), cell differentiation-inducing (Matsuda et al.1994) and enzyme inducing (Choudhury et al.1987) and inhibiting activity (Choudhury et al.1987). Diterpenoids comprise approximately 11 percent of the methanol extract of *Andrographis paniculata* (Handa and Sharma, 1990b).

SUMMARY OF THE INVENTION

The present invention involves the discovery of a group of compounds that can cause expression of $p34^{cdc2}$ kinase to decrease within a cell or group of cells. Extracts of dried plants were assayed for the ability to decrease expression of $p34^{cdc2}$ kinase. An extract of *Andrographis paniculata* was discovered to have this effect. Later analysis showed that it is the andrographolide compounds in the extract that causes the reduction of expression of $p34^{cdc2}$ kinase.

Prior work has shown that extracts of *Andrographis paniculata* and relatively pure andrographolide are safe to humans and they have been used for a variety of medicinal properties. However, prior to the present invention, it was unknown that extracts of *Andrographis paniculata* and/or andrographolide compounds could be used to lower expression of $p34^{cdc2}$ kinase. The compound has the following structure:



wherein,

R1, R2 and R5 are one of the following: a hydroxyl group, a methyl group, a methoxy group, a methylene group, or an ether or ester linked sugar group;

R3 is a methyl group or a methylene group;

R4 is a hydroxyl group or a carbonyl group;

R6 is hydrogen or a hydroxyl group.

Andrographolide is known to have the following naturally occurring analogs: 14-epiandrographolide; isoandrographolide; 14-deoxy-12-methoxyandrographolide; 12-epi-14-12-methoxyandrographolide; 14-deoxy-12-hydroxyandrographolide; and 14-deoxy-11-hydroxyandrographolide.

This discovery has led to a series of novel methods for using these extracts and/or andrographolide compounds. After angioplasty the smooth muscle cells in the arteries and veins can proliferate and restrict flow. By administering an andrographolide compound to a human patient undergoing an angioplasty procedure, this over proliferation of smooth muscle cells can be avoided. Andrographolide is effective at lowering expression of $p34^{cdc2}$ kinase in cancer cells. Lowering $p34^{cdc2}$ kinase expression can inhibit HIV-1 mediated cell death for patients with AIDS. Lowering $p34^{cdc2}$ kinase expression can inhibit phosphorylation of amyloid precursor proteins which can delay the onset of Alzheimer's

These any other diseases that respond to treatments that affect $p34^{cdc2}$ kinase activity or expression can now be treated using the teaching of the present invention.

BRIEF DESCRIPTION OF THE DRAWING

Figures 1a and 1b show schematic representations of the role of $p34^{cdc2}$ in cell division.

Figure 2 shows a dose-response curve demonstrating the effect of the methanol extract of *Andrographis paniculata* on the hepatoma test cell (Wud23) wherein the median effective concentration for the downregulation of $p34^{cdc2}$ was 22 µg/mL in this experiment.

Figure 3 shows a dose-response curve demonstrating the effect of TGFß on the concentration of $p34^{cdc2}$ protein in the hepatoma test cell (Wud23) wherein the median effective concentration of TGFß was 0.007 µg/mL.

Figure 4 shows a dose-response curve demonstrating the effect of the methanol extract of *Andrographis paniculata* on three prostate cancer cell lines wherein the median effective concentrations of andrographolide were 22, 43 and 30 µg/mL for LNCaP, PC-3 and DU-145 cells, respectively.

Figure 5 shows a dose-response curve demonstrating the effect of andrographolide on the concentration of $p34^{cdc2}$ protein in the hepatoma test cell (Wud23) wherein the median effective concentration of andrographolide in this experiment was 5 μg/mL.

Figure 6 shows the structure of andrographolide wherein the R groups indicate positions modified in naturally occurring analogs.

Figure 7 shows the effect of andrographolide at various in vitro levels and human blood levels.

Figure 8 demonstrates the dose-response relationship for andrographolide inhibition of MCF-7 cell growth.

Figure 9 shows an anti-phosphotyrosine immunoblot of MCF-7 cell lysates following 24 hours of exposure to andrographolide.

Figure 10 shows the effect of andrographolide on cyclin-dependent kinases CDK2 and CDK4 and cyclin D1.

Figure 11 shows an immunoblot of $p34^{cdc2}$ in MCF-7 whole cell lysates at 24 and 48 ours following exposure to 5 μg andrographolide/mL.

Figure 12 shows an anti-cyclin B immunoblot of cyclin B in MCF-7 whole cell lysates after exposure to 5 μg andrographolide/mL.

Figure 13 shows an anti-Rb protein immunoblot demonstrating the effect of andrographolide on the status of Rb protein phosphorylation in MCF-7 cell lysates after 48 hours of exposure.

Figure 14 shows an anti-CDK7 immunoblot indicating that andrographolide in concentrations up to 10 μg/mL had no effect on the concentration of CDK7 protein in MCF-7 cell lysates.

Figure 15 shows an immunoblot of c-Mos nuclear expression in MCF-7 cell lysate following 72 hours of exposure to 5 μg andrographolide/mL.

Figure 16 shows a proposed model for the role of c-mos action on cyclin B phosphorylation status and protein concentration.

Figure 17 shows an immunoblot of A431 cell cytosol after 24 hours of treatement with andrographolide.

Figure 18 shows a dose-response relationship for andrographolide and inhibition of HIV-1 replication.

Figure 19 shows synergy demonstrated between AZT and andrographolide in the inhibition of HIV-1 replication.

DETAILED DESCRIPTION

General Screening of Extracts of Dried Plants for the Capacity to Downregulate $p34^{cdc2}$ Concentrations

As previously described, several pathologic conditions provoke cells to over express the cyclin dependent kinases as well as cyclins. It is becoming increasingly apparent that regulation of the concentration of $p34^{cdc2}$ is important in the control of cellular proliferation (cancer and angiogenesis), as well as in the cytopathicity of AIDS, Alzheimer's and hepatitis.

Figures 1a and 1b show schematic representations of the role of $p34^{cdc2}$ in cell division. In normal resting cells, $p34^{cdc2}$ exists at low concentrations. When stimulated to divide the concentration of $p34^{cdc2}$ increases dramatically in the cell. Cancer cells are characterized by high concentrations of $p34^{cdc2}$ compared to resting cells. Stimulation of $p34^{cdc2}$ expression begins a sequence of events that in normal cells results in the elimination of enzymatically active $p34^{cdc2}$ kinase. This sequence of events is mediated through p53 and p21.

The bioassay used here to screen the methanol extracts of Dried Plants is not a measure of the kinase activity associated with the $p34^{cdc2}$ enzyme as described by others(Ducommun and Beach, 1990). This kinase activity, usually measured with histone

H1 as the substrate, is maximal at the G2/M transition and is associated with the $p34^{cdc2}$/cyclin B complex. Rather the $p34^{cdc2}$ assay used in the following examples measures the extent of expression of the holoenzyme, reflecting progression through G1 and the G1/S transition.

A variety of signal transduction pathways can theoretically affect the expression of $p34^{cdc2}$ protein in the cell. Therefore the assay used in the examples is not specific as a screen for chemicals modifying the transcription of $p34^{cdc2}$. The assay does, however, provide an excellent multi-functional screen for the capacity of a test chemical or extract of a natural product to decrease the cellular concentration of the $p34^{cdc2}$ protein. The use of this assay as a biological endpoint increases the probability of identifying compounds in complex libraries with unique mechanisms of action.

Three-hundred methanol extracts of dried plants were tested for their ability to downregulate the $p34^{cdc2}$ content of a model hepatoma cell line. Only ten percent of these extracts demonstrated a dose-related decrease in $p34^{cdc2}$ of greater than 80 percent at the highest dose tested (50 µg/mL), while demonstrating no toxicity to the cell. Identification of the active constituents from these thirty plant extracts was done by chemical fractionation and by literature searchers for secondary plant metabolites identified in these plants. Additional testing was then performed on isolated fractions as well as compounds identified through the literature.

EXAMPLE 1

The Downregulation of $p34^{cdc2}$ Protein or Growth Inhibition in Animal and Human Carcinoma Cell Lines by a Methanol Extract of *Andrographis paniculata*

Summary

A methanol extract of the leafy portion of the plant *Andrographis paniculata* was tested for the capacity to downregulate $p34^{cdc2}$ protein or inhibit cell growth in an animal hepatoma cell line and in human breast, and liver cancer cells. In each system the extract demonstrated both the ability to downregulate the $p34^{cdc2}$ protein or to inhibit cell growth over seven days. The activity of the extract was dose-related and median effective

concentrations were lowest for breast cancer cells and highest for woodchuck hepatoma cells. Both downregulation of the $p34^{cdc2}$ protein and inhibition of growth occurred at concentrations that produced no visible signs of cytotoxicity.

Materials and Methods

Methanol Extract of *Andrographis paniculata*: The leafy portion of the plant *Andrographis paniculata* was harvested in Zhejing province in the Peoples Republic of China during the summer. The harvested material was allowed to air dry until the leaves crumbled to the touch. A course powdery material was produced from the dried plant and extracted with boiling methanol under reflux for 24 hours. This extraction procedure was repeated and the two methanol solutions were pooled and the solvent was evaporated under vacuum at ambient temperature. The resultant powder was placed in a screw-cap vial and stored at room temperature in the dark until weighed for use.

Bioassay Procedure: The cell line to be tested was set-up at a 1:10 split in a T-75 flask in MEME + NEAA supplemented with sodium pyruvate and 10% FBS-HI (maintenance medium). Any cell line may be used for the testing. The cell lines used in this example were an hepatocellular carcinoma derived from a Woodchuck (designated Wud23), MCF-7 (human breast), HepG2 (human liver) LNCAP (human prostate). All human cells were obtained from American Type Culture Collection (Bethesda, MD). Approximately 4 to 5 days later (80 to 90% confluence) the cells were harvested, counted and adjusted to 2×10^4 cells/mL in maintenance media with 0.5% FBS-HI. Cells were plated (1×10^3 cells/well) in a 96-well flat bottom tissue culture plate with 50 mL media/well. The plant extract (dissolved in DMSO) was added to the wells at doses of 50, 10, 1, 0.5, and 0.1 mg/mL in 50 mL of maintenance medium with 0.5% FBS-HI. Plates were incubated at 5% CO_2, 37C, and 95% humidity for 48 hours (woodchuck hepatoma) or 7 days (human breast and liver cells) and then harvested.

Positive and negative controls: TGFß was used as a positive control as an example of a chemical known to specifically downregulate $p34^{cdc2}$ concentrations; vincristine and methotrexate were administered to the cell cultures as negative controls chemicals whose mechanism of action does not involve direct downregulation of $p34^{cdc2}$ levels.

Analysis of $p34^{cdc2}$ content of the cells: The $p34^{cdc2}$ content of the cells was quantified using an ELISA assay. Results were tabulated as fmol $p34^{cdc2}/10^3$ cells plated. The $p34^{cdc2}$ content of the treated cells was compared to the $p34^{cdc2}$ content of the six control wells run on the same plate. The percent of downregulation of $p34^{cdc2}$ observed in the test wells (triplicate) at each dose was computed relative to the controls on the same plate.

Microtiter Assay for $p34^{cdc2}$ protein:

Commercially obtained antibodies: The polyclonal antibody to $p34^{cdc2}$ kinase was obtained from Pederson Biotech Resources (Ithaca, NY), and alkaline phosphatase or peroxidase-conjugated anti-rabbit IgG were obtained from commercial sources (e.g. Transduction Laboratories, Lexington, KY; Oncogene Science, Inc. Manhasset, NY; Sigma, St. Louis, MO).

Chemicals and Materials:

Immobilon 2 microtiter plates	Dynatech (Shantilly, VA)
Bovine serum albumin (BSA)	Sigma #A-3350 (St Louis, MO)
Triton X-100	Sigma #X-100
EGTA	Sigma #E-4378
PMSF	Sigma #p-7626
Leupeptin	Sigma #L-2884
Soy bean trypsin inhibitor	Sigma #T-9003
N-tosyl-L-phenylalanine chloromethyl ketone	Sigma #T-4376
Sodium fluoride	Sigma #S-6521
b-glycerophosphate	Sigma #G-6626
Paranitrophenyl phosphate	Sigma #104-0
Sodium orthovanadate	Sigma #S-6508
DTT (dithiothreitol)	Sigma #D-0632
MgCl2	Sigma
ABTS	Sigma #A-1888
TRIS	Sigma
Sodium carbonate	Sigma

Reagents:

A. Sodium Carbonate buffer, 0.1 M, pH 9.6. - 1) Mix 71.3 mL of 1 M NaHCO3 and 28 mL of 1 M Na2CO3; 2) Add 800 mL dd H2O; 3) Adjust pH to 9.6 and Qs to 1.1 L

B. 10X Phosphate buffered saline, 0.15 M, pH 7.2. - 1) NaCl, 80.0 g/L; 2) KCl, 2.0 g/L; 3) Na2HP04, 11.5 g/L; 4) NaH2PO4, 2.0 g/L

C. Blocking buffer, PBS with 3% BSA - 1) 1X PBS with 3 g BSA per 100 mL

D. Washing buffer, PBS with 0.2% Triton X-100 - 1) 1 X PBS with 0.2 mL of Triton X-100 per mL

E. Prep buffer, 25 mM Tris-HCl, pH 8.0 with 10 mM MgCl2, 15 mM EGTA, 0.1% Triton X-100, 0.1 mM PMSF, 0.1 mM sodium fluoride, 60 mM b-glycerophosphate, 15 mM p-nitrophenylphosphate, 0.1 mM sodium orthovanidate, 1 mg/mL leupeptin, 10 mg/mL soybean trypsin inhibitor, 1 mg/mL aprotinin and 10 mg/mL tosyl-phenylalanine.

F. Assay buffer, 50 mM Tris-HCl, pH 7.4 with 10 mM MgCl2, 1 mM DTT.

G. Citrate buffer - 1) Add 9.6 g citric acid (MW 192.12) to 950 mL ddH2O; 2) Adjust pH to 4.0 with 5 M NaOH and store at 4C.

H. ABTS stock solution - 1) 0.5487 g ABTS to 25 mL with ddH20 and store at 4C.

I. ABTS substrate, 0.4 mM ABTS; 1) 0.05 mL ABTS; 2) 0.02 mL diluted H2O2 (0.5 M); 3) 5.0 mL citrate buffer.

Assay: Plates were washed in washing buffer and adhering cells were lysed and diluted in prep buffer and mixed 1:1 with blocking buffer. This was added to the wells of a plate and incubated for 1 hr at 4C with slow constant shaking. Plates were washed 3x with washing buffer and 1x with assay buffer. Two-hundred mL of primary antibody at a dilution of 1:1000 in blocking buffer was added to each well and incubated for 2 hr at 4C. Plates were washed 3x with washing buffer. Two-hundred mL of a peroxidase-conjugated secondary antibody at a dilution of 1:3000 in blocking buffer was added to each well and incubated for 1 hr at 4C. Wash plates 3x with washing buffer. Add 200 mL of ABTS

solution and read the color change on a microtiter plate reader once per minute for 10 minutes in kinetics mode (Botek EL312) at 415 nm.

Determination of Viable Cell Counts: Estimates of cell viability were made by visual observation and the Pro-Mega Cell Proliferation Assay Kit (Pro-Mega, Madison, WI). Standard curves of viable cell counts were used to relate color change of the reagent to cell counts.

Interpretation of the test: A positive response is defined as a dose-related decrease in $p34^{cdc2}$ content of the cells in excess of 50%. Calculation of median effective doses was performed on probit transformed percentages versus log concentration of the extract. The probit transformation was used to linearize the sigmoidal response data.

Results

The methanol extract of *Andrographis paniculata* produced a significant dose-related downregulation of $p34^{cdc2}$ protein within 48 hours in the Wud23 woodchuck hepatoma cell line as demonstrated by the data in Figure 2. The median effective concentration was approximately 30 µg/mL (average of two replicate studies). Excellent dose-related $p34^{cdc2}$ downregulation was also observed for human breast and liver cancer cells that correlated with growth inhibition. Both downregulation of the $p34^{cdc2}$ protein and inhibition of growth occurred at concentrations of extract that produced no visible signs of cytotoxicity.

TGFß caused a downregulation of $p34^{cdc2}$ protein in the Wud23 cells within 48 hours (Figure 3). Neither vincristine nor methotrexate produced a dose-related decrease in $p34^{cdc2}$ protein at concentrations that were not cytotoxic.

These results indicate that a compound(s) in the methanol extract of *Andrographis paniculata* is capable of downregulating the $p34^{cdc2}$ content of several transformed cell lines representing human and animal cells. It was also demonstrated that the downregulation of $p34^{cdc2}$ protein in the woodchuck hepatoma Wud23 was predictive of $p34^{cdc2}$ downregulation and inhibition of cell growth in human breast and liver cancers.

EXAMPLE 2

The Inhibition of Growth of Prostate Cancer Cells in vitro by a Methanol Extract of *Andrographis paniculata*

Summary

A methanol extract of the leafy portion of the plant *Andrographis paniculata* was tested for the capacity to inhibit cell growth in three human prostate cancer cell lines, LNCaP, PC-3 and DU-145. Median effective doses for the three cell lines, respectively, were 22, 43 and 30 µg/mL. This inhibition was achieved with no visible signs of cytotoxicity.

Methods

Human Tumor Cell Lines: Human prostate adenocarcinoma cell lines LNCap, PC-3 and DU-145 were purchased from the American Type Tissue Culture Collection. All cell lines were propagated as monolayers in RpMI-1640 containing 5% FCS, 5% NuSerum IV, 20 nM HEPES, 2 mM L-glutamine at 37C in a 5% CO2 humidified atmosphere. The doubling times for the cell lines ranged between 34 and 42 hours.

Growth Inhibition Assay in 96 Well Microtiter Plates: Cells were plated between 1400 cells/well (PC-3 and Du-145) and 3000 cells per well (LNCaP) in 96 well plates and incubated at 37C 48 hour prior to drug addition to allow attachment and exponential growth of cells. The methanol extract of *Andrographis paniculata* was solubilized in 100 % DMSO and further diluted in RPMI-1640 containing 10 mM HEPES. Each cell line was treated with 10 concentrations of extract ranging from 30 µg/mL to 1 ng/mL. After a 72 hour (PC-3, DU-145) or 120 hour (LNCaP) incubation, 100 mL of ice-cold TCA was added to each well and incubated for 1 hour at 4C. Plates were then washed 5 times with tap water to remove TCA, low-molecular weight metabolites and serum proteins. Fifty mL of 0.4% sulforhodamine B (SRB) was added to each well. Following a five minute incubation at room temperature, plates were rinsed 5 times with 0.1 % acetic acid and air dried. Bound dye was solubilized with 10 mM Tris Base (pH 10.5) for 5 minutes on a gyrating shaker. Optical density was measured at 570 nm.

Results

Median effective doses for LNCaP, PC-3 and DU-145, respectively, were 22, 43 and 30 µg/mL (Figure 4). This inhibition was achieved with no visible signs of cytotoxicity. These results are consistent with the findings of downregulation of $p34^{cdc2}$ protein in the Wud23 hepatocellular carcinoma cell line; Over two experiments this cell line exhibited a median effective doses of 22 and 40 µg/mL. Over four cell lines representing both animals and humans, the methanol extract of *Andrographis paniculata* has demonstrated the ability to downregulate the cell division control protein $p34^{cdc2}$ kinase. Furthermore, EXAMPLES 1 and 2 demonstrate that the capacity of a substance to downregulate $p34^{cdc2}$ kinase in tumor cells correlates with the inhibition of growth of tumor cells. This result is mechanistically sound since the movement through the cell cycle is controlled and coordinated by $p34^{cdc2}$ kinase.

EXAMPLE 3

The Downregulation of $p34^{cdc2}$ Protein in an Hepatocellular Carcinoma Cell Line by Andrographolide

Summary

The diterpenoid lactone andrographolide was tested for the capacity to downregulate $p34^{cdc2}$ protein in an animal hepatoma cell line. In this test system andrographolide was able to downregulate the $p34^{cdc2}$ protein in a dose-related manner within 48 hours. The median effective concentrations was estimated from a log-probit regression as 5.0 µg/mL. Downregulation of the $p34^{cdc2}$ protein occurred at concentrations that produced no visible signs of cytotoxicity.

Materials and Methods

Andrographolide: Andrographolide was purchased from Aldrich Chemicals (Milwaukee, WI) and provided at a purity of 98 percent.

Bioassay Procedure: The procedure described in EXAMPLE 1 was used with only Wud23 cells and the exposure period was 48 hours. Concentrations of

andrographolide tested included 50, 25, 12.5, 10, 5, 2,5, 6.25, 1.25, 0.625, 0.5, 0.25, 0.125, 0.1, 0.05, 0.025 and 0.0123 mg/well.

Analysis of $p34^{cdc2}$ content of the cells: The $p34^{cdc2}$ content of the cells was quantified using an ELISA assay as described in EXAMPLE 1.

Determination of Viable Cell Counts: Estimates of cell viability were made by visual observation.

Interpretation of the test: A positive response was defined as a dose-related decrease in $p34^{cdc2}$ content of the cells in excess of 50%.

Results

Andrographolide produced a significant dose-related downregulation of $p34^{cdc2}$ protein within 48 hours in the Wud23 woodchuck hepatoma cell line as demonstrated by the data in Figure 5. The median effective concentration was approximately 5.0 mg/mL or 6 times more potent than the methanol extract (30 mg/mL).

These results indicate that andrographolide was capable of downregulating the $p34^{cdc2}$ content of transformed cells. Since the increase in potency demonstrated by andrographolide in this bioassay was in proportion to its concentration in the methanol extract of *Andrographis paniculata*, it is reasonable to assume that the downregulation of $p34^{cdc2}$ by the herbal extract was due to andrographolide and its diterpeniod analogs.

EXAMPLE 4

Downregulation and Inhibition of Phosphorylation of $p34^{cdc2}$ and Cyclin B (MPF) in MCF-7 Human Breast Cancer Cells by Andrographolide Is Mediated via Mos

Summary

The diterpenoid lactone andrographolide was tested for its capacity to affect cell growth, protein tyrosine phosphorylation, and expression and phosphorylation status of cell cycle regulatory proteins in the MCF-7 human breast cancer cell line. In this human test system andrographolide was able to inhibit cell growth. The median effective

inhibitory concentration was estimated to be 0.82 μg andrographolide/mL (IC_{50} 95% Confidence Interval = 0.32 to 1.3 μg/mL). Cell cycle progression in asynchronous cells was inhibited within 48 hours of exposure to 5 μg andrographolide/mL with an increase in G1 phase cells from 56% at zero time to 68 and 73% at 48 and 72 hours, respectively.

Tyrosylphosphoproteins at 85, 45 and 33 kDa were completely eliminated from anti-phosphotyrosine immunoblots of cell lysates of MCF-7 cells treated with 5 μg/mL of andrographolide for 24 hours. Neither CDK2 or CDK4 concentrations in whole cell lysates were affected at 5 or 10 μg andrographolide/mL over the 72 hours of the experiment. Cyclin D1 exhibited a dose-related decrease in concentration in whole cell lysate at 5 and 10 μg andrographolide/mL following 72 hours of exposure.

Concentrations and phosphorylation status of both $p34^{cdc2}$ and cyclin B, the components of MPF (maturation promoting factor) were reduced at 24 and 48 postdosing hours at 5 μg andrographolide/mL. Relative to the control, concentrations of 5 or 10 μg andrographolide/mL exhibited a dose-related decrease in the extent of phosphorylation of Rb protein with increasing drug. Andrographolide in concentrations up to 10 μg/mL for 24 hours had no effect on the concentration of CDK7 protein in MCF-7 cell lysates.

At 5 μg andrographolide/mL, expression of c-Mos forms p35, p37 and p40 in the nuclear fraction of MCF-7 cells was decreased 48% in total at 72 postdosing hours. The effect of andrographolide on Mos concentrations offers an explanation for the decrease in cyclin B protein concentrations and may be the critical cellular target of andrographolide. This is the first demonstration of a decrease in intracellular Mos protein concentrations by a low molecular weight chemical. Although overexpression of Mos proteins have been implicated in cellular transformation, no experiments before this have shown a relationship between a decrease in Mos protein and inhibition of cancer cell growth.

This EXAMPLE teaches that labdane diterpenes such as andrographolide can function to decrease the intracellular concentration of Mos proteins and that Mos proteins can serve as a target to control the growth of cancer cells. Since the only known constitutive function of Mos proteins is in meiosis, compounds that selectively inhibit Mos proteins to control cancer growth would be expected to exhibit low toxicity to somatic cells and to affect only oocyte maturation and spermatogenesis.

Methods

Chemicals - All antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Andrographolide was synthesized by Aldrich Chemicals (Milwaukee, WI) and provided at a purity greater than 98 percent. Ribonuclease was purchased from Worthington Biochemical Corp, Freehold, NJ). All other chemicals were purchased from Sigma (St. Louis) and were the highest purity commercially available.

Human Breast Cancer Cell Line - The human breast adenocarcinoma cell line MCF-7 was purchased from the American Type Tissue Culture Collection. The cell line was propagated in Eagle's minimum essential medium with non-essential amino acids and 5% serum. The MCF-7 cell line retains several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and is capable of forming domes. The MCF-7 cells were cultured at 37°C in a 5% CO_2 balanced air environment. Cells were subcultured by centrifuging (1000 X g for three minutes) and suspending in 100 mL of fresh media to a cell density of 5.0×10^5 cell/mL.

Bioassay Procedure - The procedure described in EXAMPLE 1 was used with MCF-7 cells and the exposure periods were 24, 48 and 72 hours. Concentrations of andrographolide tested included 0, 0.05, 0.5, 0.63, 1.25, 2.5, 5 and 10 µg/mL. After the incubation period, cells were harvested by centrifugation and washed three times in PBS (phosphate buffered saline) at 4°C and lysed in 20 mM Tris buffer (pH 8.0) with 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethyl-sulfonyl fluoride, 0.15 units/mL of aprotonin, and 1 mM sodium orthovanadate at 4°C for 20 minutes.

Determination of Median Inhibitory Concentration (IC_{50}) - Growth of the MCF-7 breast carcinoma was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product (Mossman, T. Rapid colorimetric assay for cellular growth and survival: Applications to proliferation and cytotoxicity assays. J. Immunol Methods 65:55-63 (1983).). MCF-7 cells were plated at initial densities of 1000 cells per well in 100 L of medium in 96-well plates. One-hundred µL of an andrographolide solution was added at twice the final concentration in dimethyl sulfoxide (DMSO) and medium. Control cells received only 100 µL of the DMSO medium solution without drug. The

formazan product of MTT reduction was dissolved in DMSO, and absorbance was measured using an MR5000 microtiter plate reader (Dynatech Laboratories Corp, Chantilly, VA). Absorbance at 570 nm was converted to cells per well based upon a standard curve developed from visual cell counts and MTT reduction. Quantification of viable cells was performed when control wells reached approximately 80 to 90 % confluence (usually within seven days). Percent inhibition of cell growth relative to controls was plotted versus log dose and estimates of median inhibitory concentrations of andrographolide were made graphically. Four replicates of the dose-response experiment were performed on each of two separate days. Estimates of the IC_{50} of andrographolide for MCF-7 cells did not differ between the two separate determinations.

Flow Cytometry - Nuclei were isolated directly from adherent MCF-7 cells using 25 mM HEPES containing 0.4% Nonidet P-40 as described by Koss et al. (1989) (Koss, L.G., Czerniak, B., Herz, F. and Wersto, R.P. Flow cytometric measurements of DNA and other cell components in human tumors: a critical appraisal. Hum Pathol 20:528-548 (1989).). Nuclei were incubated with ribonuclease (500 U/mL) at room temperature for one hour and stained in the dark at 4°C for 12 - 18 hours with 50 µg/mL propidium iodide. DNA content was measured using a FACScan (Becton Dickinson, San Jose, CA) flow cytometer. Data acquisition and analysis were carried out using CellFit software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Statistical analysis of cell cycle variables was done using chi-square analysis (JMP Version 2 Software, SAS Institute, Inc., Cary, NC).

Gel Electrophoresis and Immunoblotting - SDS-PAGE was carried out as described in Laemmli and Favre, 1973, (Laemmli, U.K. and M. Favre. Maturation of the head of bacteriophage T4. I. DNA packaging events. J Mol.Biol. 80:575-599 (1973).) using 7.5 - 10% polyacrylamide gels with the modification that MCF-7 cell lysates (100 µg protein/lane) were subjected to heat treatment (100°C) for eight minutes. The immunoblotting was carried out as described by Towbin et al. (1979) (Towbin, H., T. Staehelin and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc.Natl.Acad.Sci.U.S.A. 76:4350-4354 (1979).); however a Milliblot SDE electroblot apparatus (Millipore, Bedford, MA), was used to transfer proteins from polyacrylamide gels to an Immobilon

membrane filter (Millipore, Bedford, MA). Complete transfers were accomplished in 25-30 min at 500 mA. Membrane filters were blocked by incubating in TBS (50 mM Tris, 150 mM NaCl, pH 7.5) containing 5% commercial nonfat dry milk for 30 min at room temperature and incubated 2 hour with 5 µg/mL antibody in TBST (0.05% Tween 20 in TBS). Molecular weights of immunostained proteins were estimated by adding molecular weight standards to reference lanes and by staining the membrane filters with amido Back 10 B. To visualize the antibody reactions, the membranes were incubated for 2 hours at room temperature with alkaline phosphatase-conjugated anti-rabbit IgG diluted 1:1000 in TBST and developed for 15 minutes. For quantification, the immunoblots were translated into TIFF-formatted files using a Microtech 600GS scanner and quantified using Scan Analysis software (BIOSOFT, Cambridge, UK).

Results

Median Inhibitory Concentration (IC_{50}) of Andrographolide in MCF-7 Cells: Figure 8 demonstrates the dose-response relationship for andrographolide inhibition of MCF-7 cell growth. In this human breast cancer test system, andrographolide was able to inhibit growth of MCF-7 cells. The median inhibitory concentration was estimated to be 0.82 µg/mL and the 95% confidence interval from the four replicates within one day was 0.32 to 1.3 µg andrographolide/mL.

Flow Cytometry: Cell cycle progression was inhibited within 48 hours of exposure to 5 µg andrographolide/mL with an increase in G1 phase cells from 56% at zero time to 68 and 73% at 48 and 72 hours, respectively (Table 4.1).

Table 4.1

The effect of andrographolide on progression through the cell cycle in non-synchronous MCF-7 cells

	Time Post Exposure (hours)			
Percent of Cells	0	24	48	72
In G1 Phase	56	58	68	73
In S Phase	20	18	14	7
In G2/M Phase	24	24	18	20

The effect of andrographolide on progression through the cell cycle in synchronous MCF-7 cells is presented in Table 4.2.

Table 4.2

The effect of andrographolide on progression through the cell cycle in synchronous MCF-7 cells

	Andrographolide (g/mL)					
Percent of Cells	**0**	**0.5**	**5**	**10**	**20**	**40**
In G1 Phase	43	49	55	63	69	76
In S Phase	35	31	27	21	13	7
In G2/M Phase	25	23	21	18	21	19

Twenty-four hours after plating, growth medium was removed and the MCF-7 cells were maintained in medium containing 0.1% serum for 48 hours. Following this period in low-serum medium, Andrographolide was added at 0, 0.5, 5, 10, 20 or 40 µg/mL in replacement medium containing 20% serum. Twenty-four hours after serum stimulation, cells were harvested and nuclei were examined by flow cytometry. A dose-related increase was seen in the percentage of cells remaining in G1 [A] following serum stimulation. The decrease in the percentage of cells in S phase [B] mirrored the increases percentage of cells in G1. Relatively little change was seen in the percentage of cells in G2/M [C]. Cells treated at 40 µg/mL survived the 24-hour exposure, but cell counts revealed 37 percent fewer cells at the higher doses than in controls. This was most likely due to the inhibition of cell division observed at the higher concentrations of andrographolide.

Immunoblotting

Anti-phosphotyrosine - Figure 9 shows an anti-phosphotyrosine immunoblot of MCF-7 cell lysates following 24 hours of exposure to andrographolide. As seen in Figure 9, lane C, there were a total of 31 tyrosylphosphoproteins present in the MCF-7 whole cell lysate. Lanes A and B, respectively 10 and 5 µg andrographolide/mL, indicate that ten of these 31 tyrosylphosphoproteins were reduced by andrographolide treatment. The apparent molecular weights of the tyrosylphosphoproteins affected by andrographolide were 91, 87, 85, 79, 69, 53, 45, 33, 19, and 12 kDa. Those tyrosylphosphoproteins at 85, 45 and 33 kDa were completely eliminated within 24 hours at the 5 µg

andrographolide/mL concentration [lane B]. Tyrosylphosphoproteins at 116, 102, 74, 51, 26, 25, 17 and 14 kDa representing major staining bands were not affected by either of the two concentrations of andrographolide over the 72-hour period of the experiment.

CDK2, CDK4 and cyclin D1 - Figure 10 shows the effect of andrographolide on cyclin-dependent kinases CDK2 and CDK4 and cyclin D1. The immunoblots indicate no effect of andrographolide on CDK2 or CDK4 expression at 5 [B] or 10 [A] μg/mL over the 72 hours of the experiment. Cyclin D1 expression, however, demonstrated a dose-dependent decrease.

$p34^{cdc2}$ - Figure 11 shows an immunoblot of $p34^{cdc2}$ in MCF-7 whole cell lysates at 24 and 48 ours following exposure to 5 μg andrographolide/mL. Both the concentration and phosphorylation status of $p34^{cdc2}$ were reduced at 24 and 48 postdosing hours in MCF-7 whole cell lysates exposed to 5 μg andrographolide/mL. As seen in Figure 11, control samples exhibited three staining bands with the anti-$p34^{cdc2}$ antibody. The lowest band represents the nonphosphorylated form, while the two upper bands represent increasing phosphorylation. Within 24 hours, a decrease in the extent of phosphorylation of $p34^{cdc2}$ can be readily observed as a loss in the staining intensity of the hyperphosphorylated band compared to controls. At 48 hours both upper bands representing phosphoprotein had nearly disappeared and a decrease was noted in the major staining band as well.

Cyclin B - Figure 12 shows an anti-cyclin B immunoblot of cyclin B in MCF-7 whole cell lysates after exposure to 5 μg andrographolide/mL. As seen with $p34^{cdc2}$, both the concentration and phosphorylation status of cyclin B were reduced at 24 and 48 postdosing hours in MCF-7 whole cell lysates exposed to 5 μg andrographolide/mL (Figure 12). However unlike $p34^{cdc2}$, it was the nonphosphorylated form (lower band) of cyclin B than was more demonstrably affected at the 24-hour time period.

Rb protein - Figure 13 is an anti-Rb protein immunoblot demonstrating the effect of andrographolide on the status of Rb protein phosphorylation in MCF-7 cell lysates after 48 hours of exposure. Relative to the control, concentrations of 5 or 10 μg andrographolide/mL exhibited a dose-related decrease in the extent of phosphorylation of Rb protein with increasing drug.

CDK7 - The anti-CDK7 immunoblot in Figure 14 indicates that andrographolide in concentrations up to 10 μg/mL for 24 hours had no effect on the concentration of CDK7 protein in MCF-7 cell lysates. This result indicates that the decrease in phosphorylation status of $p34^{cdc2}$ was not due to a decrease in the expression or concentration of the CDK7 component of CAK.

Mos - Figure 15 shows an immunoblot of c-Mos nuclear expression in MCF-7 cell lysate following 72 hours of exposure to 5 μg andrographolide/mL. At this test concentration, expression of c-Mos forms p35, p37 and p40 in the nuclear fraction of MCF-7 cells was decreased 48% in total at 72 postdosing hours.

Interpretation

The most dramatic and rapid biochemical changes that occurred with the treatment of MCF-7 cells with andrographolide were the decreases in expression and phosphorylation status of $p34^{cdc2}$ and cyclin B. Neither expression nor apparent phosphorylation status of CDK2, CDK4 or CDK7 were affected by andrographolide. Therefore it is reasonable to conclude that the decrease in Rb phosphorylation observed was due to effects of andrographolide on $p34^{cdc2}$ and cyclin B, since Rb is an identified substrate of all CDK/cyclin protein complexes. Any interpretation of the mechanism of action of andrographolide must explain the effects on $p34^{cdc2}$ and cyclin B phosphorylation.

The data in Figures 12 and 13 offer a model for the action of andrographolide in the inhibition of tumor cell proliferation. It has been proposed that c-Mos functions to prevent degradation of cyclin B by maintaining it in a highly phosphorylated form (Roy, L.M., Singh, B., Gautier, J., Arlinghaus, R.B., Nordeen, S.K. and Maller, J.L. The cyclin B2 component of MPF is a substrate for the c-mosxe proto-oncogene product. Cell 61:825-831 (1990).). Inhibition of this function would result in a decrease in amount of phosphorylated cyclin B as well as a loss of nonphosphorylated cyclin B due to rapid degradation of the nonphosphorylated form. These effects are seen with andrographolide in Figure 12. The loss of c-Mos expression is depicted in Figure 15. This decrease in c-Moss expression may be explained if andrographolide functions to inhibit the

autophosphorylation of c-Mos. Nonphosphorylated, inactive c-Mos would also be targeted for degradation.

A model for the interpretation of these results is presented in Figure 16. Inhibition of c-Mos phosphorylation of cyclin B results in a loss of cyclin B protein through the rapid degradation of nonphosphorylated cyclin B. As presented in this EXAMPLE, the nonphosphorylated form of cyclin B (lower band) was absent in MCF-7 cell lysate from andrographolide-treated cells within 24 hours. By 48 hours the continued inhibition of c-Mos phosphorylation of cyclin B results in further loss of the cyclin B pool and concomitant decrease of phosphorylated cyclin B. The reduction of cyclin B phosphorylation seen in these experiments is similar to the reduction observed when c-Mos is ablated from oocytes by injection of c-Mos antisense oligonucleotide (Roy et al., 1990).

EXAMPLE 5

Andrographolide Downregulates Mos Associated Proteins in A431 Human Epidermal Cells

Summary

The diterpenoid lactone andrographolide was tested for its capacity to affect the expression of Mos associated proteins in A431 Cells. In this human epidermoid carcinoma test system andrographolide was able to significantly alter the degree of expression of c-Mos and v-Mos associated proteins within 24 hours. EC_{50} values of 0.28, 0.74 and 0.68 μg andrographolide/mL were obtained, respectively, for c-Mos associated proteins p35, p37, and p40. v-Mos associated proteins p29 and p43 exhibited EC_{50} values for downregulation of 6 and 3 μg andrographolide/mL, respectively.

Methods

Chemicals: Anti-Mos antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Andrographolide was purchased from Aldrich Chemicals (Milwaukee, WI) and provided at a purity greater than 98 percent. All other chemicals

were purchased from Sigma (St. Louis) and were the highest purity commercially available.

Human Epidermoid Cancer Cell Line: The human epidermoid carcinoma cell line A431 was purchased from the American Type Tissue Culture Collection. The cell line was propagated in Dulbecco's modified Eagle's medium with 4.5 g/L glucose, 90%; fetal bovine serum, 10%. The cell line was originally derived from an 85-year old female. The A431 cells were cultured at 37°C in a 5% CO_2 balanced air environment. Cells were subcultured by centrifuging (1000 X g for three minutes) and suspendening in 100 mL of fresh media to a cell density of 5.0×10^5 cell/mL.

Bioassay Procedure: The procedure described in EXAMPLE 1 was used with A431 cells and the exposure period was 24 hours. Concentrations of andrographolide tested included 0, 0.05, 0.5, 5 and 10 mg/mL. After the 24 hour incubation period, cells were harvested by centrifugation and washed three times in PBS (phophate buffered saline) at 4°C and lysed in 20 mM Tris buffer (pH 8.0) with 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethyl-sulfonyl fluoride, 0.15 units/mL of aprotonin, and 1 mM sodium orthovanadate at 4°C for 20 minutes.

Gel Electrophoresis and Immunoblotting for Mos Proteins: SDS-PAGE was carried out as described (Laemmli and Favre, 1973) using 7.5 - 10% polyacrylamide gels with the the modification that cell lysates (100 mg protein/lane) were subjected to heat treamtent (100°C) for eight minutes. The immunoblotting was carried out as described by Towbin et al. (1979); however a Milliblot SDE electroblot apparatus (Millipore, Bedford, MA), was used to transfer proteins from polyacrylamide gels to an Immobilon® membrane filter (Millipore, Bedford, MA). Complete transfers were accomplished in 25-30 min at 500 mA. Membrane filters were blocked by incubating in TBS (50 mM Tris, 150 mM NaCl, pH 7.5) containing 5% commercial nonfat dry milk for 30 min at room temperature and incubated 2 hour with 5 mg/mL anti-Mos in TBST (0.05% Tween 20 in TBS). Molecular weights of immunostained proteins were estimated by adding molecular weight standards to reference lanes and by staining the membrane filters with amido Back 10 B. To visualize the antibody reactions, the membranes were incubated for 2 hour at room temperature with alkaline phosphatase-conjugated anti-rabbit IgG for CDK1 or anti-mouse IgG for PCNA diluted 1:1000 in TBST and developed for 15 minutes. For

quantification, the immunoblots were translated into TIFF-formattted files using a microtech 600GS scanner and quantified using Scan Analysis software (BIOSOFT, Cambridge, UK).

Results

Figure 17 shows an immunoblot of A431 cell cytosol after 24 hours of treatement with andrographolide. A431 cell lysate showed five immunoreactive bands with anti-Mos antibody (Figure 17). These bands exhibited apparent molecular weights of 43, 40, 37, 35 and 29 kDa. All of the immunoreactive bands appeared as doublets in controls indicating phosphorylation. p40, p37 and p35 immunoreactive bands were consistent with c-Mos forms, while immunoreactive bands at p43 and p29 were consistent with v-Mos forms.

Table 5.1 - Andrographolide inhibition of Mos protein expression in A431 cells in 24 hours

				EC50	
Mos Forms	r	Slope	Intercept	µg/ml	µM
p29	0.996	1.009	4.187	6	18
p35	0.939	0.834	5.46	0.28	0.80
p37	0.998	1.143	5.151	0.74	2.11
p40	0.994	1.114	5.19	0.68	1.93
p43	0.998	1.343	4.377	3	8

Andrographolide was able to significantly alter the degree of expression of c-Mos and v-Mos associated proteins within 24 hours. EC_{50} values of 0.28, 0.74 and 0.68 mg andrographolide/mL were obtained, respectively, for c-Mos associated proteins p35, p37, and p40. v-Mos associated proteins p29 and p43 exhibited EC_{50} values for downregulation of 6 and 3 mg andrographolide/mL, respectively (Table 5.1).

EXAMPLE 6

Andrographolide Inhibits HIV-1 Replication and Synergizes AZT Inhibition of HIV-1 Replication in Human Peripheral Monocytes Infected *in vitro* with HIV-1

Summary

Andrographolide is a naturally occurring diterpene lactone that functions biochemically as a kinase inhibitor. Andrographolide demonstrated the capacity to inhibit the replication of HIV-1 in peripheral blood monocytes (PBMC) as measured by the degree of inhibition of reverse transcriptase (RT) activity. The IC_{50} of andrographolide for this effect was 640 ng/mL. Toxicity of andrographolide to normal, noninfected PBMC was not observed at concentrations of andrographolide of 10,000 ng/mL, providing a minimum therapeutic index in excess of 15-fold (10,000/640). Further testing of andrographolide indicated that andrographolide synergized with AZT in the inhibition of HIV-1 replication in PBMC as measured by reverse transcriptase activity.

Although reverse transcriptase activity was used as a marker for the replication of the HIV-1WEJO virus, reverse transcriptase is not considered the site of action of the test compound andrographolide. The previous EXAMPLES have demonstrated that andrographolide inhibits the phosphorylation of $p34^{cdc2}$ kinase. Evidence to date, included in previous examples, indicates that this may be due to the inhibition of c-Mos activation by andrographolide which in turn would result in the inhibition of the phosphorylation of Mek-1 (Pham et al., 1995).

Since Mek-1 activation results in the increase in phosphorylation of $p34^{cdc2}$, inhibition of Mek-1 phosphorylation would inhibit HIV-1 cytopathicity in a manner described by Cohen et al. (Science, 1992, 256:542-545). The cellular target of andrographolide in the inhibition of cancer cells and HIV-1, however, appears logically to stem upstream of $p34^{cdc2}$ phosphorylation to inhibition of c-Mos activation. As the putative target for andrographolide, c-Mos inhibition affords a mechanistic explanation for the effects of andrographolide on both $p34^{cdc2}$ and cyclin B1. Furthermore, the inhibition of HIV-1 replication infers that inhibition of c-Mos is critical in the control of HIV-1 induced cytopathicity of infected T-cells.

This EXAMPLE teaches that inhibition of critical cellular serine/threonine kinases, such as c-Mos allow for the control of HIV-1 replication in normal, human PBMC. As such, it is the first demonstration that this group of kinases (serine/threonine) are critical in the replication of the AIDS virus. This cellular target may have utility in the control of

other viral infections that result in cytopathicity as a result of overexpression of $p34^{cdc2}$ or cyclin B1 and the stimulation of the cell cycle.

Materials & Methods

Chemicals: Andrographolide was synthesized by Aldrich as previously referenced (EXAMPLE 5). 3'-deoxy-3'azidothymidine (AZT) was obtained from Burroughs Wellcome (Research Triangle Park, NC). All other standard laboratory chemicals were purchased from commercial suppliers as described in previous EXAMPLES or as indicated and were of the highest purity available.

Cells and viruses: H9 cells were used to propagate HIV-1WEJO and these reagents were obtained through the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases, Bethesda, MD. Cells were maintained in RPMI 1640 medium containing 10% fetal calf serum and 40 IU of interleukin-2 per mL (for PBMC) in a humid 5% CO_2 atmosphere. For propagation of HIV-1WEJO, fresh human mononuclear cells were prepared from the blood of healthy donors by Ficoll gradient centrifugation. The cells were suspended in RPMI 1640 medium containing 5% human a type AB+ serum and were cultivated on hydrophobic membranes (Teflon bags; $3x10^6$ cells per mL). After 24 hours the PBMC were infected by the addition of HIV-1WEJO-infected lymphocytes ($5x10^4$ infected PBMC per mL). Forty-eight hours after infection, the cells were transferred to 24-well plates (Falcon), and the nonadhering cells were quantitatively removed. The adherent cells were further grown in a culture volume of 1.3 mL, and fresh medium was added every week.

Determination of Median Inhibitory Concentrations (IC_{50}): A total of 105 cells were infected with virus stocks at 0.01 PFU per cell, seeded onto 24-well plates and incubated for 72 to 96 hours in the presence of 0, 0.0671, 0.2097, 0.6554, 2.0480, 6.4, or 20 µg andrographolide/mL dissolved in dimethyl sulfoxide (DMSO) and diluted in cell culture medium. For a positive control, infected cells were also incubated with 0, 0.0034, 0.0105, 0.0328, 0.1024, 0.3200 or 1.0 µg AZT/mL.

Reverse Transcriptase Assay: In the endogenous reverse transcriptase assay, wherein the viral RNA functioned as the template, the reaction mixture (50 µL) consisted

of 50 mM Tris-HCl (pH 8.4), 2.5 mM MgCl2, 100 mM KCl, 4 mM dithiothreitol, 30 μg of bovine serum albumin per mL, 0.5 mM EGTA [ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid], and 0.01% (wt/vol) Triton X-100. Of the four deoxynucleoside triphosphates, three were used at a saturating concentration of 100 μM, while the tritium-labeled dGTP (Amersham) was used at a concentration of 2.5 μM. Specific activity was 11 Ci/mmol. Percent inhibition of reverse transcriptase relative to controls was plotted versus log dose and estimates of median inhibitory concentrations of andrographolide were made using linear regression of the probit of the percent inhibition versus log dose. Two replicates of the dose-response experiment were performed. Estimates of the IC_{50} of andrographolide for inhibition of HIV-1 replication in PBMC did not differ between the two separate determinations.

For the determination of cytotoxic concentrations, media containing dilutions of the compounds dissolved in DMSO were added to wells containing 2.5 x 10^5 cells and the mixtures were incubated. The number of viable cells were determined after 96 hours of cultivation using the neutral red assay. Synergy of AZT and Andrographolide

The combined effect of two compounds on HIV-1WEJO replication in PBMC was studied under the experimental conditions described for the median effective concentration determinations. A dilution matrix with two-fold dilutions was prepared in four individual 96-well plates. On the basis of the three-dimensional surface diagram, volumes of synergy and antagonism at 95% confidence limits were calculated by using the MacSynery II program 1.0 (C. Shipman, University of Michigan, Ann Arbor). According to the author of this program, synergy and antagonism volumes under 25 (μg/mL)% should be regarded as nonsignificant. Values between 25 and 50 (μg/mL)% indicate minor, but significant synergy. Values over 50 or 100 (μg/mL)% indicate moderate or intense synergy or antagonism, respectively.

Neutral Red Assay for Viable Cells: The following solutions were prepared in advance of the assay:

Neutral Red 1.0% in 0.1 M Na_2HPO_4 (in foil wrapped bottle). This solution was stored at refrigerator temperature (4°C) and is stable for up to six months.

UPTAKE SOLUTION: RPMI:EBSS (1:1) with 2% fetal bovine serum, 0.05 M HEPES pH 7.1 (0.2 µm filtered sterilized). Prepare in advance and store in the refrigerator up to six months.

WASH: 0.85% NaCl. Prepare in advance and store at room temperature up to one year.

EXTRACTION: 0.1 M Na_2HPO_4:Ethanol (1:1). Prepare 0.1 M Na_2HPO_4 in advance and store up to six months at room temperature. Mix with ethanol on the day of the assay at room temperature. Discard unused mixture.

Prepare uptake solution in water bath to approximately 37°C (five minutes). Add neutral red solution to appropriate amount of required uptake solution to give approximately 0.05% concentration of neutral red. This would involve a 1:20 dilution of neutral red into uptake solution. Remove RPMI growth media from the wells of the culture plates by inverting the plates followed by gentle blotting on absorbent towels resting on plastic wrap to prevent leakage on the benchtop. Mix uptake solution and under reduced lighting (use only indirect lighting in the area of work) add 100 µL to each well for media control, sample dilution or virus control. Cover and incubate at 37°C with 5% CO_2 and humidity for one hour. Record the results on a microplate reader in the dual mode with a 10 nm band pass filter at 550 nm and a 10 nm band pass reference filter at 620 nm.

Results

Median Inhibitory Concentration (IC50) of Andrographolide against HIV-1WEJO: In this in vitro human HIV-1 test system, andrographolide was able to inhibit growth of HIV-1WEJO cells as measured by inhibition of reverse transcriptase activity. The median inhibitory concentration was estimated to be 640 ng/mL. The dose-response relationship for andrographolide inhibition of HIV-1WEJO cell growth is presented in Figure 18. The graph represents the probit of the percent of inhibition of reverse transcriptase in human PBMC infected with HIV-1WEJO.

Synergy of AZT and Andrographolide: As seen in Figure 19, at the lower range of concentration tested for AZT and andrographolide there was synergy was demonstrated in excess of 50%. At higher concentrations of both drugs antagonism was observed. The antagonistic effect of the two drugs at the higher concentrations tested may be due to the fact that reverse transcriptase was used as the response variable in this study. Based upon mechanistic studies in tumor cells, andrographolide would inhibit viral replication at a pre-reverse transcriptase stage.

Toxicity of Andrographolide to Normal PBMC: Overt toxicity of andrographolide to normal, noninfected PBMC was not observed at concentrations of andrographolide up to 10,000 ng/mL, providing a minimum therapeutic index in excess of 15-fold (10,000/640).

Interpretation

Although reverse transcriptase activity was used as a marker for the replication of the HIV-1WEJO virus, reverse transcriptase is not considered the site of action of the test compound andrographolide. Other biochemical studies (c.f. previous EXAMPLES) have demonstrated that andrographolide inhibits the phosphorylation of $p34^{cdc2}$ kinase. Evidence to date, included in previous examples, indicates that this may be due to the inhibition of c-Mos activation by andrographolide which in turn would result in the inhibition of the phosphorylation of Mek-1 (Pham et al., 1995).

Since Mek-1 activation results in the increase in phosphorylation of $p34^{cdc2}$, inhibition of Mek-1 phosphorylation would inhibit HIV-1 cytopathicity in a manner described by Cohen et al. (Science, 1992, 256:542-545). The cellular target of andrographolide in the inhibition of cancer cells and HIV-1, however, appears logically to stem upstream of $p34^{cdc2}$ phosphorylation to inhibition of c-Mos activation. As the putative target for andrographolide, c-Mos inhibition affords a mechanistic explanation for the effects of andrographolide on both $p34^{cdc2}$ and cyclin B1. Furthermore, the inhibition of HIV-1 replication infers that inhibition of c-Mos is critical in the control of HIV-1 induced cytopathicity of infected T-cells.

This EXAMPLE teaches that inhibition of critical cellular serine/threonine kinases, such as c-Mos allow for the control of HIV-1 replication in normal, human PBMC. As such, it is the first demonstration that this group of kinases (serine/threonine) are critical in the replication of the AIDS virus. This cellular target may have utility in the control of other viral infections that result in cytopathicity as a result of overexpression of $p34^{cdc2}$ or cyclin B1 and the stimulation of the cell cycle.

EXAMPLE 7

Dose Regimens For Down Regulations of $p34^{cdc2}$

The following dose regimens would be anticipated to be effective in the downregulation of $p34^{cdc2}$ in humans based upon the information generated on *Andrographis paniculata* and andrographolide dosing in humans (Zhang et al.1994; Thamlikitkul et al.1991) and animal efficacy (Puri et al.1993; Wang and Zhao, 1993; Zhao and Fang, 1991; Zhao and Fang, 1991) and toxicity (Kapil et al.1993; Visen et al.1993; Shukla et al.1992; Akbarsha et al.1990; Handa and Sharma, 1990b; Handa and Sharma, 1990a; Zoha et al.1989) studies of the extract or pure compound. Inferences as to the dose of andrographolide necessary were made using the information that andrographolide is approximately 11 percent of a methanol extract or 0.78 percent of the total plant material (Handa and Sharma, 1990b).

The dose regimens described are suggested based only on the information developed on the absorption, distribution and efficacy or andrographolide itself. Distribution and metabolism of a chemical may be profoundly affected through the simultaneous administration of other drugs, individual genetics, age, other diseases of the patient and the progression of the disease itself. Therefore, the doses described in this section represent ideal cases and are to serve only as a guide to the physician. The importance of the continued monitoring of the patient by the physician should not be ignored. When dictated by information concerning the patients response to therapy, the physician should either decrease or increase the dose or duration of the recommended therapy.

Smooth muscle cell stenosis following angioplasty: The inhibition of smooth muscle cell proliferation following angioplasty can be achieved by dosing the patient with 1 to 15 mg andrographolide/kg body weight, three times per day starting two days before the operation and continuing for 30 days following the operation. Since phenobarbital metabolism is inhibited by the administration of andrographolide, barbiturate anesthetics should be avoided during the angioplasty.

Cancer: The inhibition of the proliferation of cancer cells can be prevented by dosing the patient with 2.5 to 15 mg andrographolide/kg body weight, two or three times per day continuously until indications of metastasis or tumor growth are negative. Due to the relative low toxicity of andrographolide demonstrated in animal studies, this dose regimen may be continued for months without interruption.

Inhibition of T-cell loss in HIV-1 mediated cell death: The inhibition of T-cell loss in HIV-1 mediated cell death can be prevented by dosing the patient with 1.0 to 10 mg andrographolide/kg body weight per day once a day continuously. The survival of the T cell is dependent upon continuous administration of andrographolide.

Inhibition of cell proliferation associated with viral infections: Cell proliferation and related cytopathicity associated with other viral diseases such as hepatitis and herpes may be prevented on a dose regimen of 2.5 to 15.0 mg andrographolide/kg body weight two to three times per day.

Inhibition of phosphorylation of amyloid precursor protein involved in the pathogenicity of Alzheimer's:

The phosphorylation of amyloid precursor protein by $p34^{cdc2}$ kinase may be prevented through the downregulation of $p34^{cdc2}$ achieved by andrographolide administration. Continuous administration of 10 mg andrographolide/kg body weight once a day will achieve the necessary degree of downregulation of $p34^{cdc2}$ in neural cells while minimizing the effect in other tissues.

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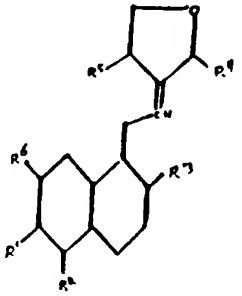
The foregoing description has been directed to particular embodiments of the invention in accordance with the requirements of the Patent Statutes for the purposes of illustration and explanation. It will be apparent, however, to those skilled in this art that many modifications and changes will be possible without departure from the scope and spirit of the invention. It is intended that the following claims be interpreted to embrace all such modifications.

What is claimed is:

1. A method of decreasing expression or phophorylation of $p34^{cdc2}$ kinase within a cell or group of cells comprising the step of administering an effective amount of a diterpene lactone compound such that said compound comes into contact with said cell or group of cells and the expression of $p34^{cdc2}$ kinase within said cell or group of cells is reduced, wherein said compound has the following structure:



wherein,

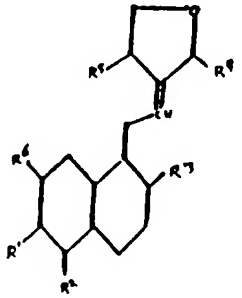
R1, R2 and R5 are one of the following: a hydroxyl group, a methyl group, a methoxy group, a methylene group, or an ether or ester linked sugar group;

R3 is a methyl group or a methylene group;

R4 is a hydroxyl group or a carbonyl group;

R6 is hydrogen or a hydrohxyl group.

2. A method of decreasing expression or phosphorylation of Mos associated proteins within a cell or group of cells comprising the step of administering an effective amount of a diterpene lactone compound such that said compound comes into contact with said cell or group of cells and the expression of $p34^{cdc2}$ kinase within said cell or group of cells is reduced, wherein said compound has the following structure:



wherein,

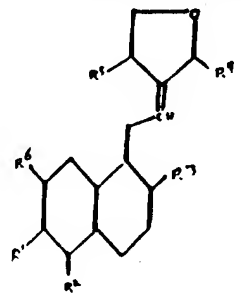
R1, R2 and R5 are one of the following: a hydroxyl group, a methyl group, a methoxy group, a methylene group, or an ether or ester linked sugar group;

R3 is a methyl group or a methylene group;

R4 is a hydroxyl group or a carbonyl group;

R6 is hydrogen or a hydroxyl group.

3. A method of decreasing expression or phosphorylation of cyclin B within a cell or group of cells comprising the step of administering an effective amount of a diterpene lactone compound such that said compound comes into contact with said cell or group of cells and the expression of $p34^{cdc2}$ kinase within said cell or group of cells is reduced, wherein said compound has the following structure:



wherein,

R1, R2 and R5 are one of the following: a hydroxyl group, a methyl group, a methoxy group, a methylene group, or an ether or ester linked sugar group;

R3 is a methyl group or a methylene group;

R4 is a hydroxyl group or a carbonyl group;

R6 is hydrogen or a hydroxyl group.

4. A method of decreasing expression or phosphorylation of $p34^{cdc2}$ kinase within a cell or group of cells comprising the step of administering an effective amount of an

extract of *Andrographis paniculata* such that said extract comes into contact with said cell or group of cells and the expression of $p34^{cdc2}$ kinase within said cell or group of cells is reduced.

5. A method of decreasing expression or phosphorylation of Mos associated proteins within a cell or group of cells comprising the step of administering an effective amount of an extract of *Andrographis paniculata* such that said extract comes into contact with said cell or group of cells and the expression of $p34^{cdc2}$ kinase within said cell or group of cells is reduced.

6. A method of decreasing expression or phosphorylation of cyclin B within a cell or group of cells comprising the step of administering an effective amount of an extract of *Andrographis paniculata* such that said extract comes into contact with said cell or group of cells and the expression of $p34^{cdc2}$ kinase within said cell or group of cells is reduced.

7. The method of claim 1 wherein said cell or group of cells are selected from the following:

a) smooth muscle cells;

b) cancer cells;

c) T-lymphocytes;

8. The method of claim 1 wherein said compound is administered at a dosage of 1 to 15 mg compound per kilogram of body of said human patient one to three times per day.

9. The method of claim 2 wherein said cell or group of cells are selected from the following:

a) smooth muscle cells;

b) cancer cells;

c) T-lymphocytes;

10. The method of claim 3 wherein said compound is administered at a dosage of 1 to 15 mg compound per kilogram of body of said human patient one to three times per day.

11. The method of claim 3 wherein said cell or group of cells are selected from the following:

a) smooth muscle cells;

b) cancer cells;

c) T-lymphocytes;

12. The method of claim 3 wherein said compound is administered at a dosage of 1 to 15 mg compound per kilogram of body of said human patient one to three times per day.

13. The method of claim 4 wherein said cell or group of cells are selected from the following:

a) smooth muscle cells;

b) cancer cells;

c) T-lymphocytes;

14. The method of claim 4 wherein said compound is administered at a dosage of 1 to 15 mg compound per kilogram of body of said human patient one to three times per day.

15. The method of claim 5 wherein said cell or group of cells are selected from the following:

a) smooth muscle cells;

b) cancer cells;

c) T-lymphocytes;

16. The method of claim 5 wherein said compound is administered at a dosage of 1 to 15 mg compound per kilogram of body of said human patient one to three times per day.

17. The method of claim 6 wherein said cell or group of cells are selected from the following:

a) smooth muscle cells;

b) cancer cells;

c) T-lymphocytes;

18. The method of claim 6 wherein said compound is administered at a dosage of 1 to 15 mg compound per kilogram of body of said human patient one to three times per day.

Figure 1a

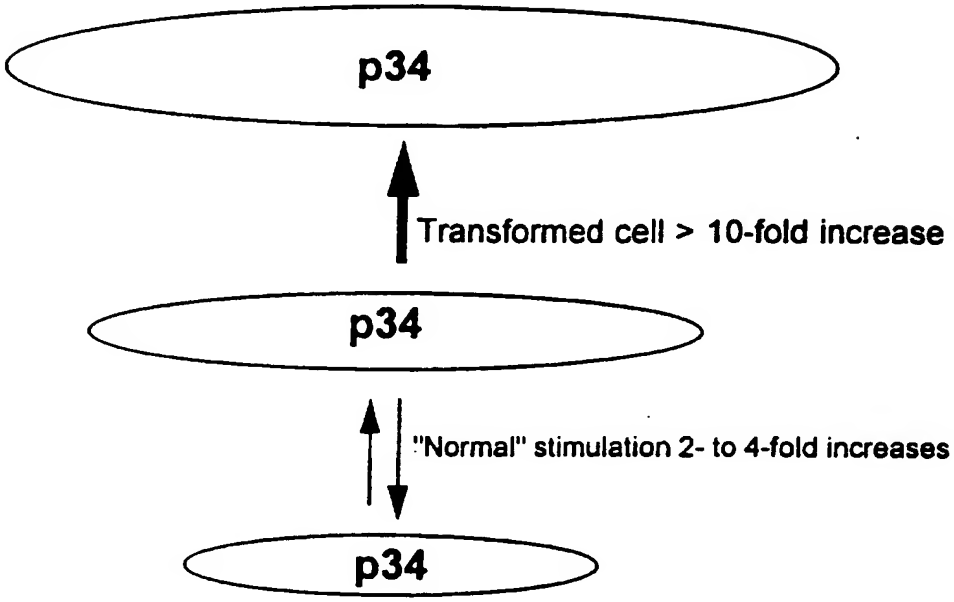


Figure 1b

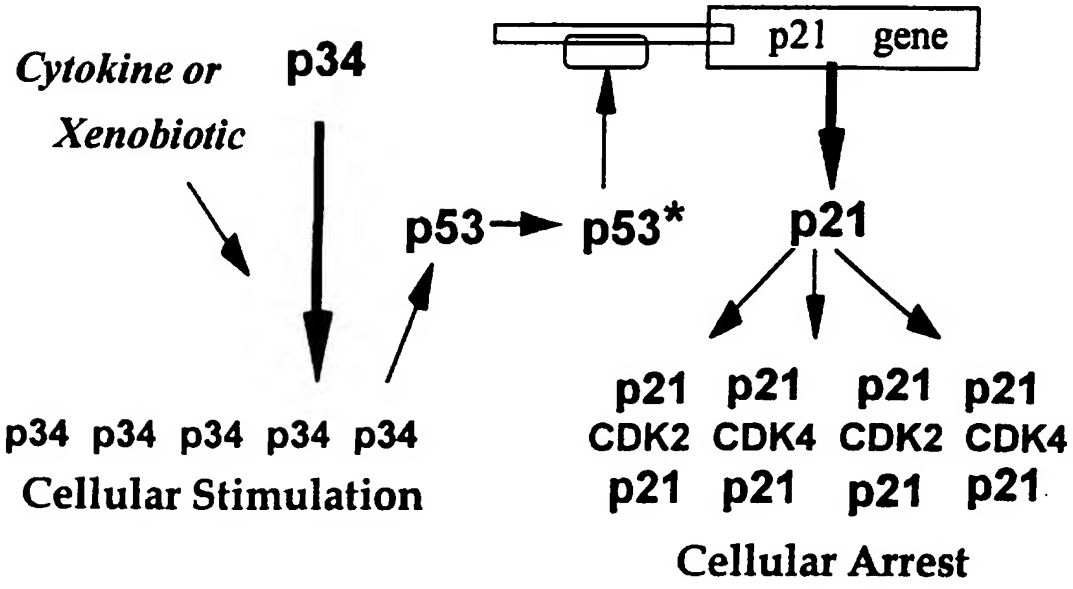


Figure 2

Data Wud23.11.16.94-#27

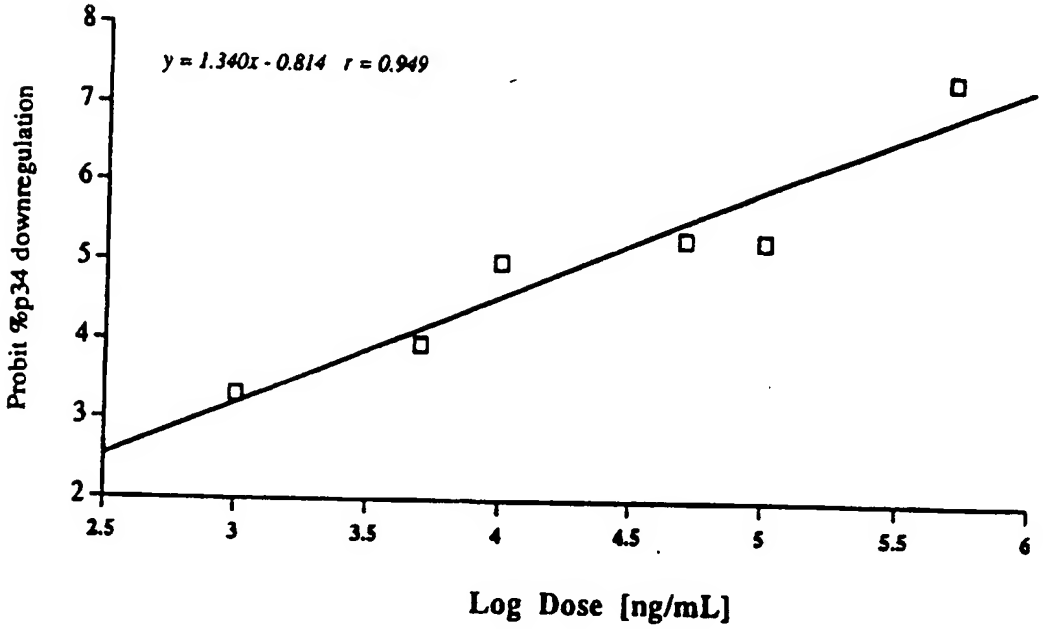


Figure 3

TGF-ß DOSE-RESPONSE CURVE - Wud23 CELLS

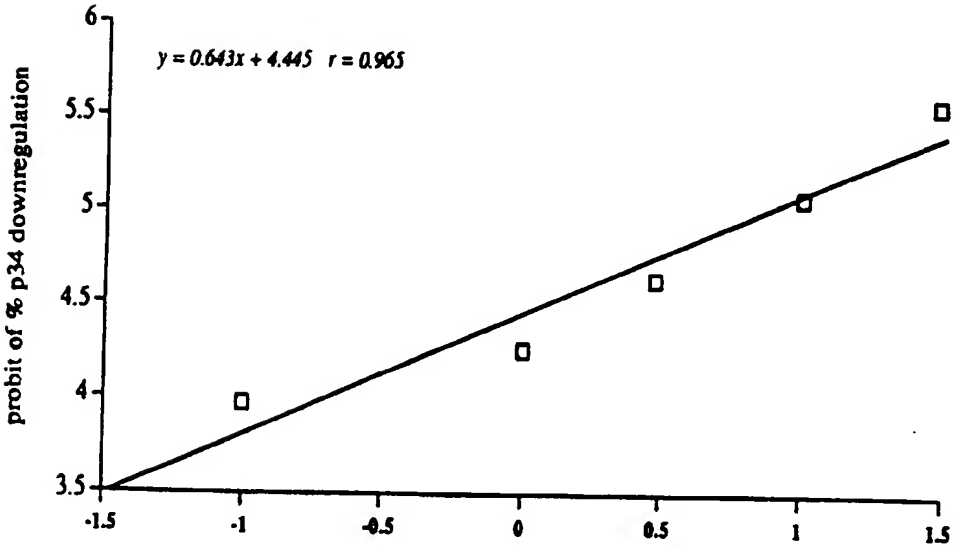


Figure 4

Effect of PC #27 on Prostate Cell Growth
(CD 94049)

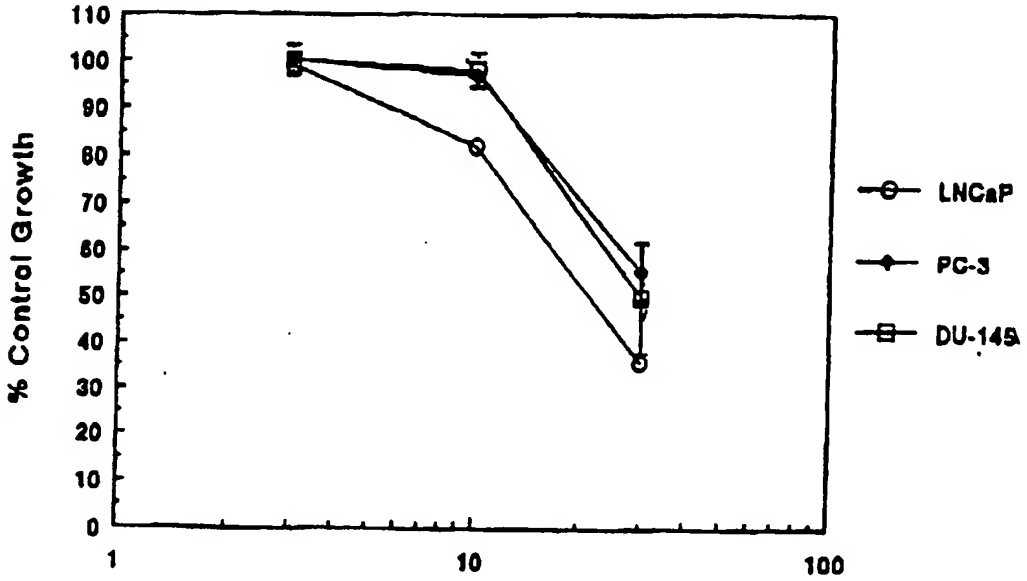


Figure 5

Data andrographilide

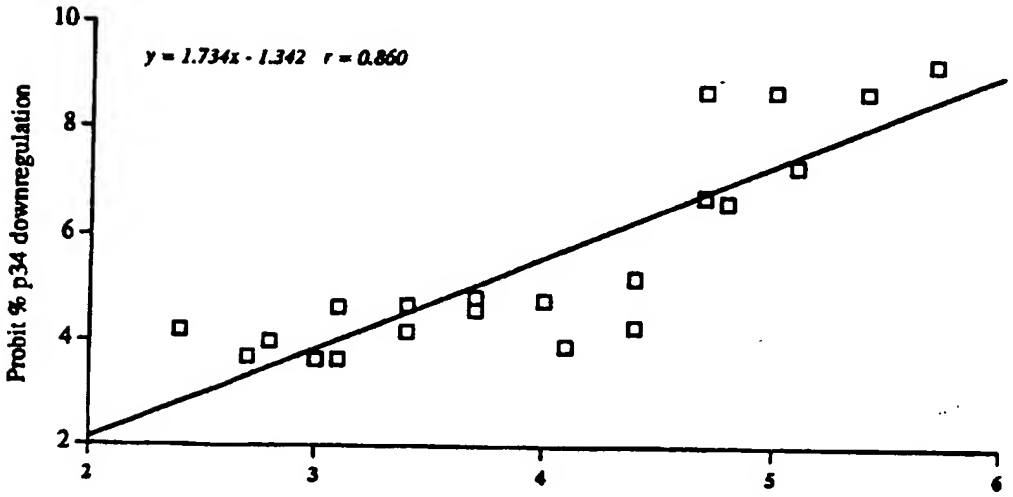


Figure 8

Inhibition of MCF-7 Cell Growth by Andrographolide

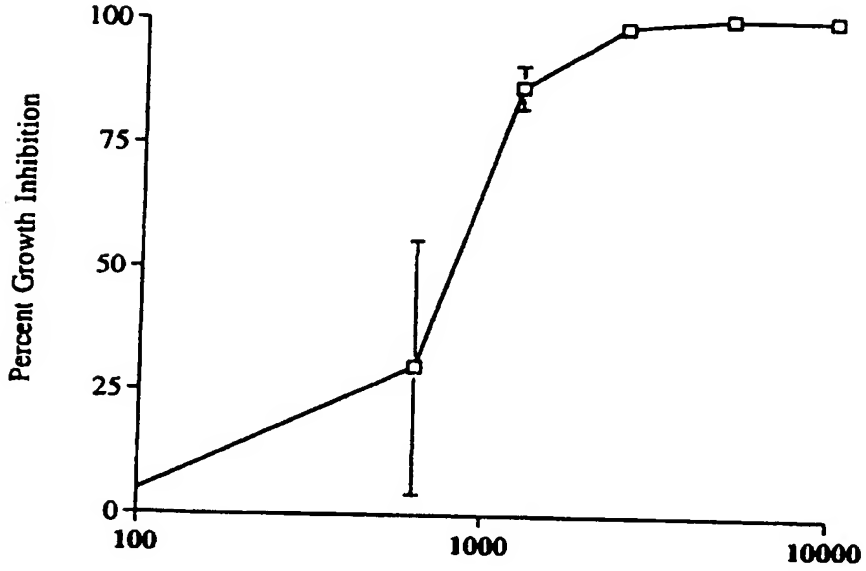


Figure 6

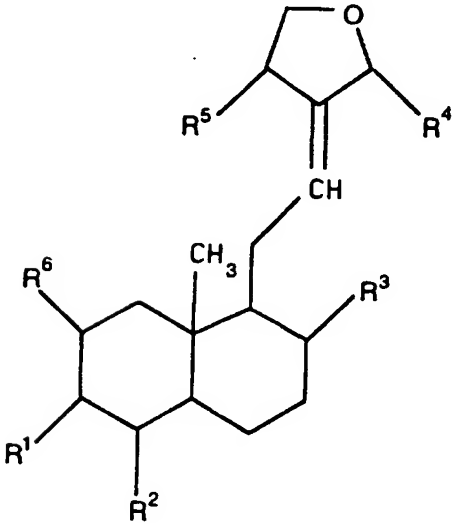


Figure 7

In Vitro EC_{50s} and Human Blood Levels [est µg/mL] of Andrographolide

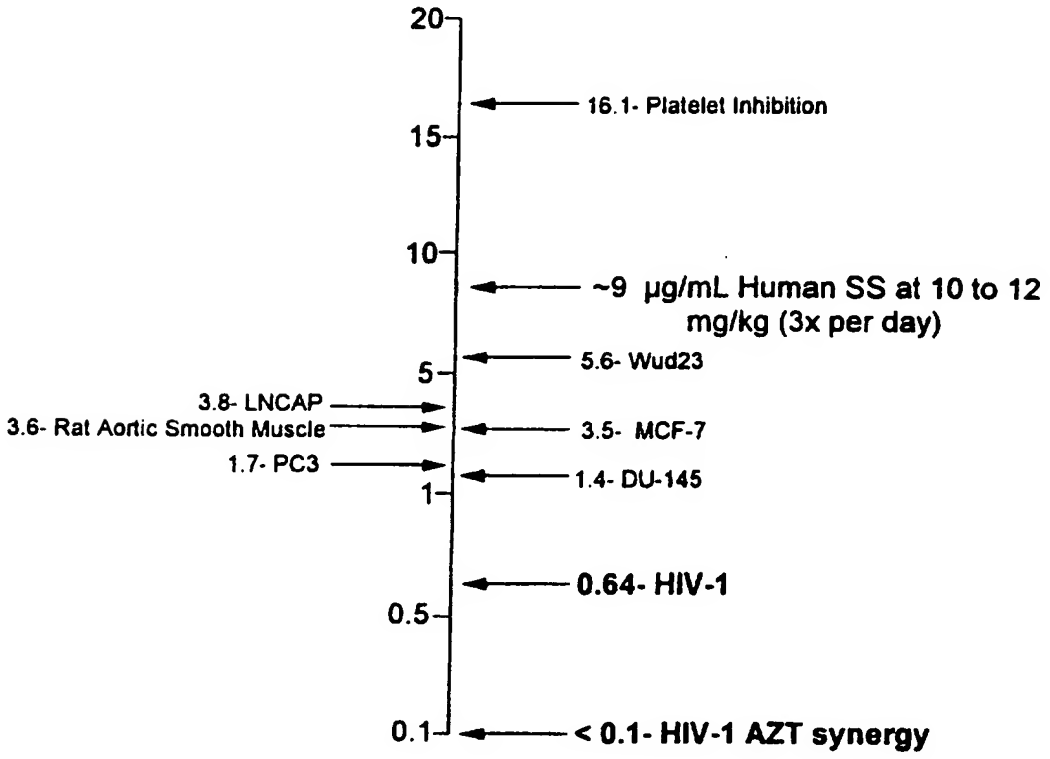


Figure 9

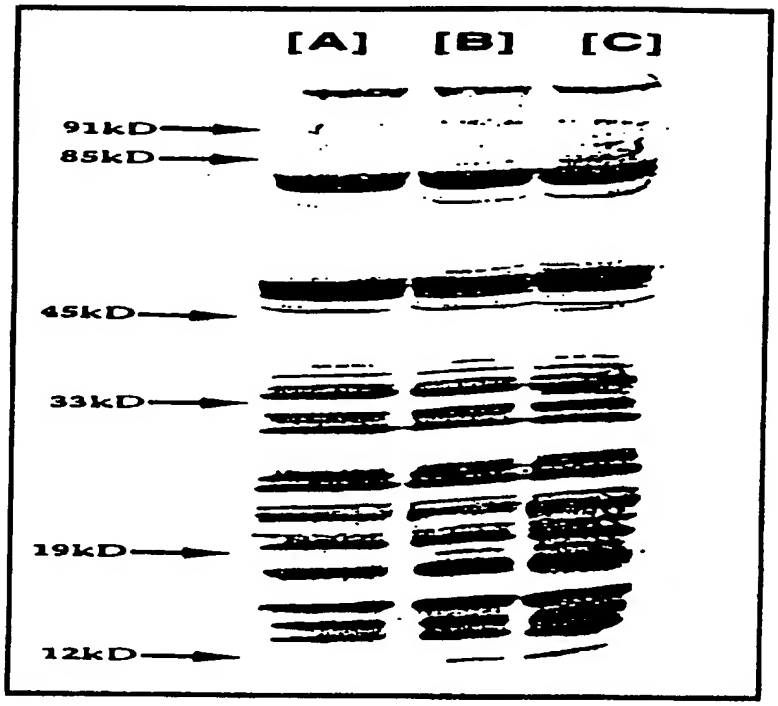


Figure 10

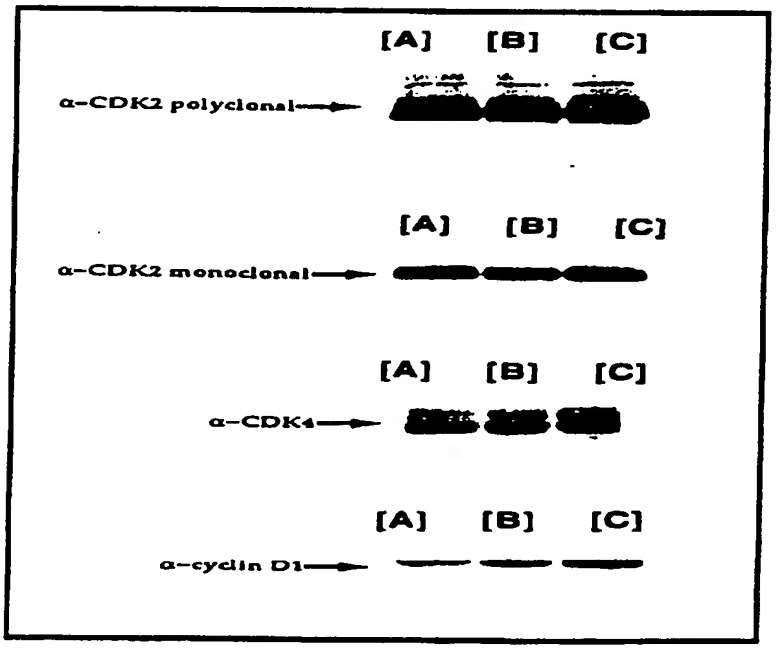


Figure 11

[MCF-7 Cell with α–p34cdc2 antibody]

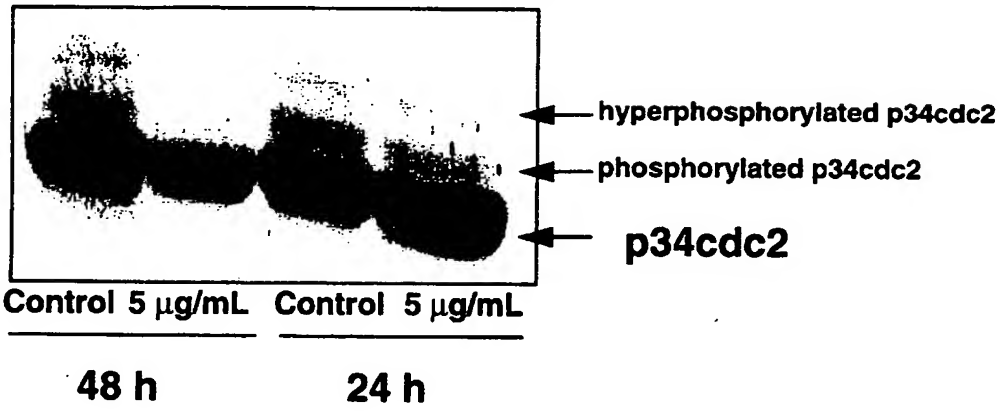


Figure 12

[MCF-7 lysate with α–cyclin B antibody]

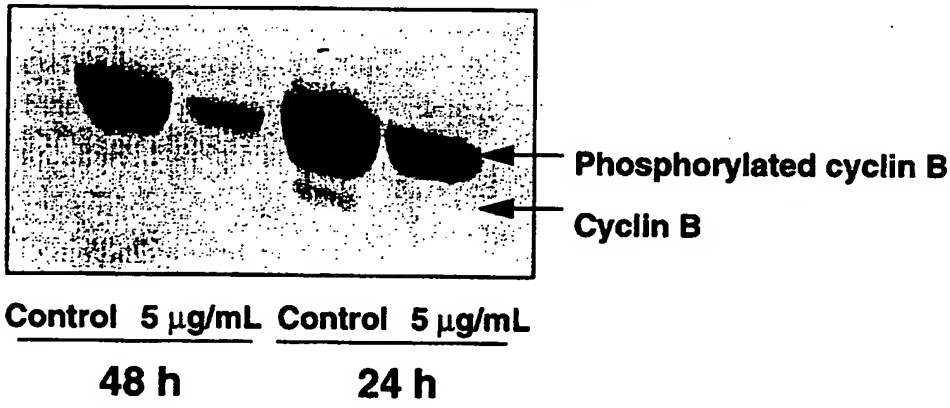


Figure 13

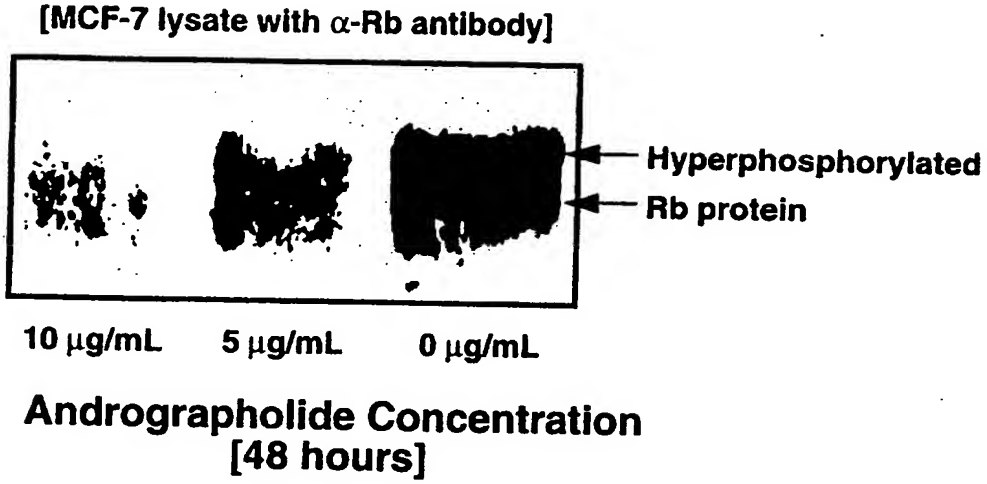


Figure 14

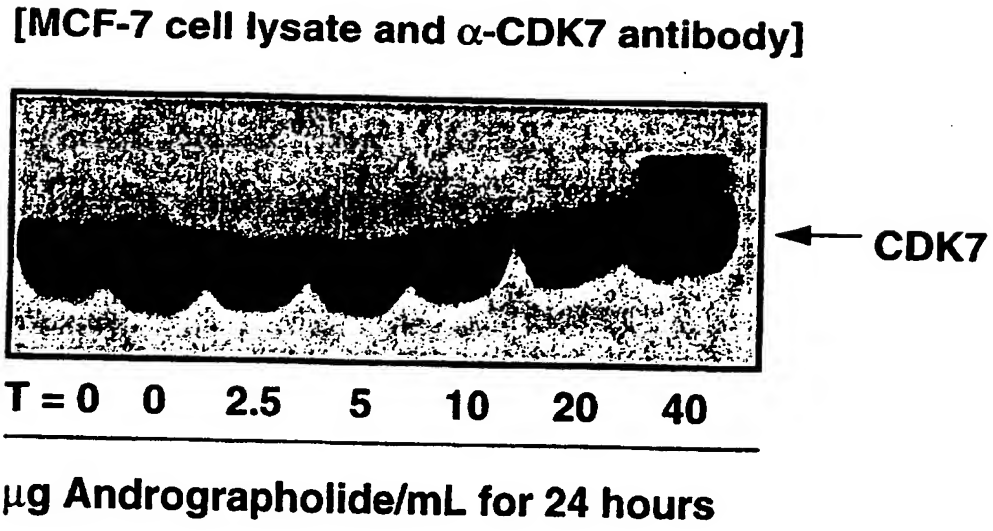


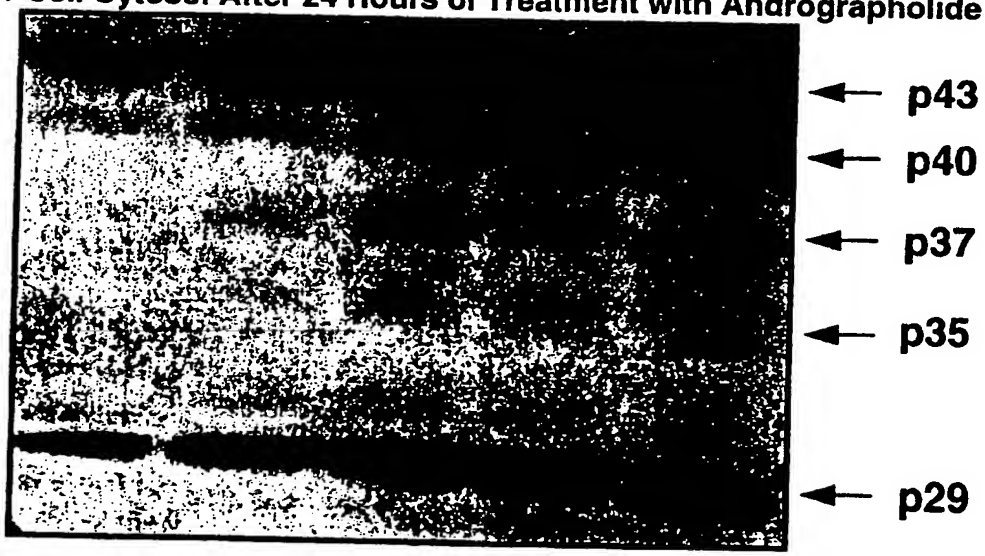
Figure 15

[MCF-7 nuclear fraction and α–cMos antibody]

post-dosing 72 hours

Figure 17

A431 Cell Cytosol After 24 Hours of Treatment with Andrographolide



µg Andrographolide/mL

Figure 16
Cyclin B Phosphorylation by Mos

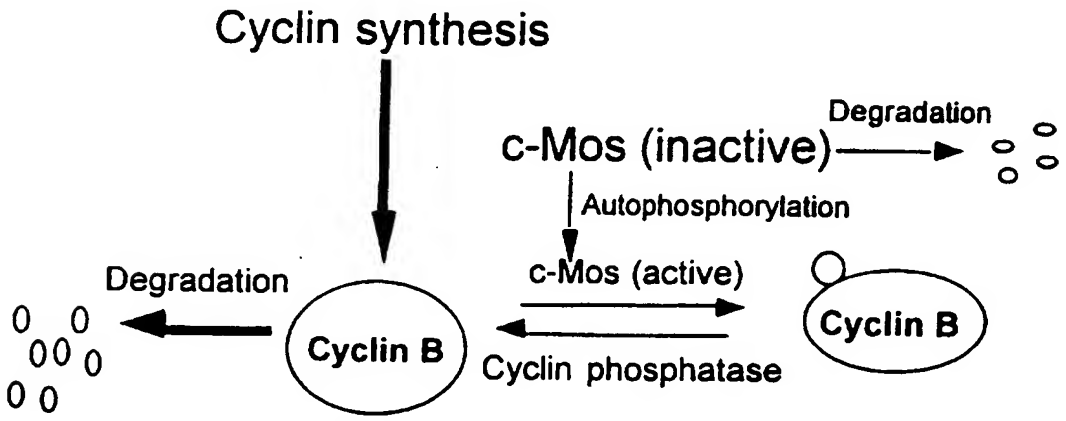


Figure 18
Inhibition of HIV-1 Replication with Andrographolide

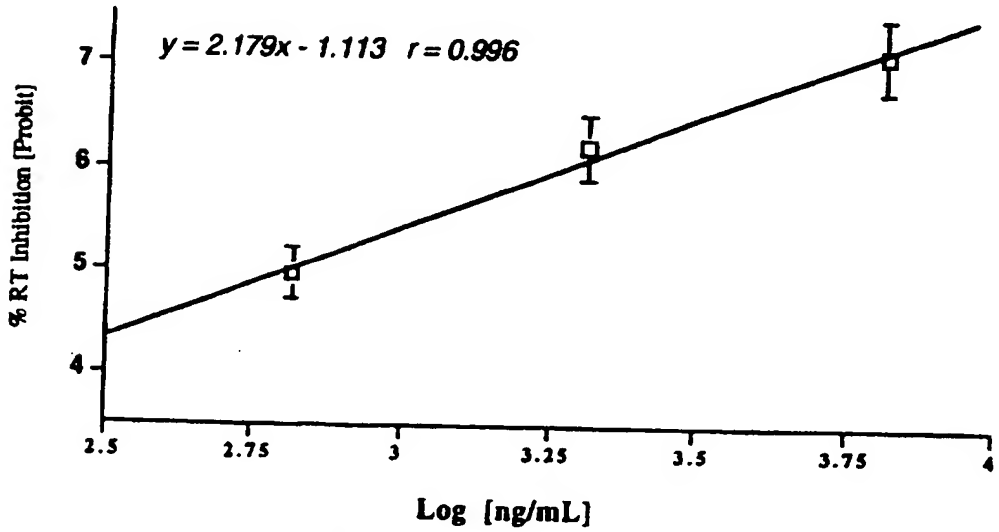
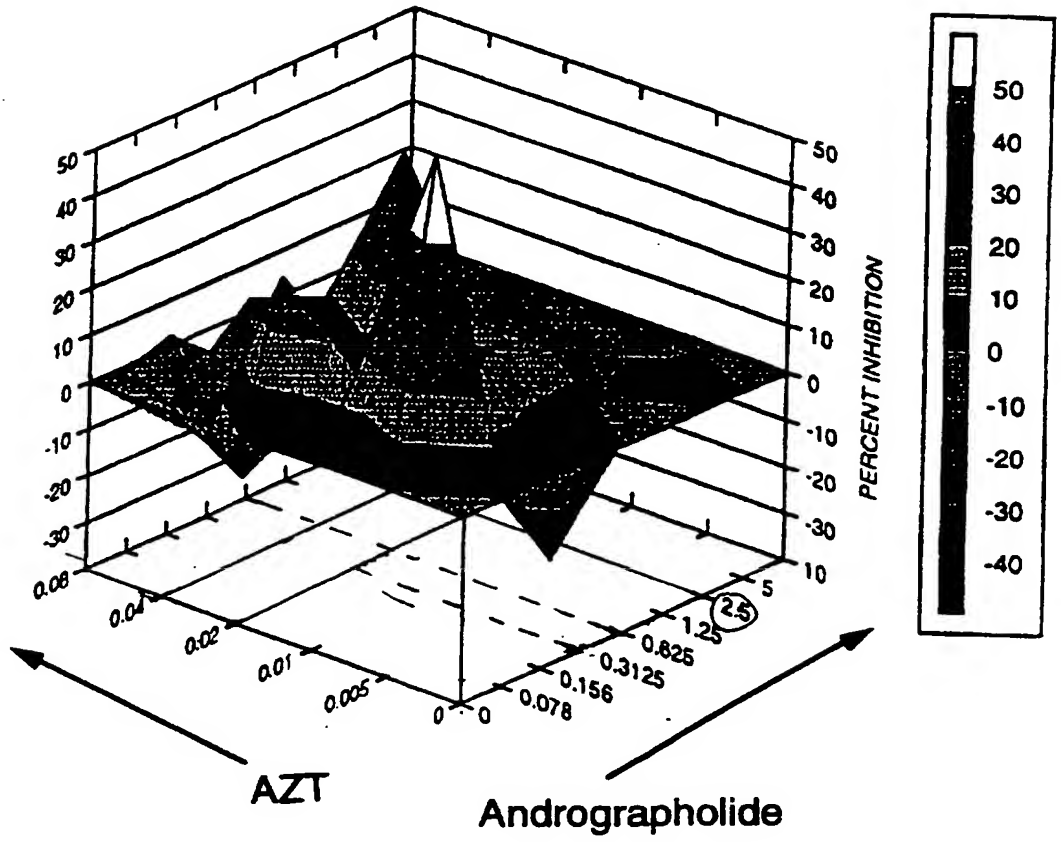


Figure 19

Anti-HIV-1_{WEJO} Activity of Andrographolide and AZT in PBMC



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/15915

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/365

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEM.PHARM.BULL., vol. 42, no. 6, June 1994, pages 1216-1225, XP000567717 "Cell differentiation-inducing diterpenes from Andrographis Paniculata Nees" see page 1216, left-hand column, line 23 - right-hand column, line 10 ---	1-18
Y	WO,A,91 01742 (RUFFLES ET AL.) 21 February 1991 see claims 1,7 --- -/--	1-18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 April 1996	03.05.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gerli, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/15915

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ADV.CONTRACEPT.DELIV.SYST., vol. 3, no. 4, 1987, pages 329-45, XP000568141 "Antifertility effects of dehydroandrographolide derivatives on mice and rats" see page 329, line 1 - line 6 see page 329, line 26 - line 34 -----	1-18

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/15915

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-18 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In' 'tional Application No
PCT/US 95/15915

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9101742	21-02-91	AU-B- 6272490	11-03-91